

**MANAGEMENT OF ACETALDEHYDE AND OTHER SO₂
BINDING COMPOUNDS TO REDUCE SULFITE
DEPENDENCY DURING VINIFICATION**

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John Nicholas Jackowetz

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John Nicholas Jackowetz, Ph.D.

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ABSTRACT

The potency of SO₂ is highest when it is in a free or unbound state. High concentrations of SO₂ binders in wine may greatly diminish the efficacy of SO₂. The decreased preservative efficacy of bound SO₂ requires increased SO₂ additions to wine for adequate preservation action. However, additional sulfite concentrations may increase the risk of adverse health reactions for sensitive consumers and may also exceed legal limits for SO₂ in wines. Hence, this work investigated important SO₂ binding compounds throughout vinification and developed strategies for their control to increase the preservative efficacy of SO₂ and limit total additions to wine.

Comprehensive analysis of SO₂ binding compounds at practical concentrations was achieved through the development of a novel UHPLC method that used the metal chelator, EDTA, to limit oxidation reactions during sample preparation. This method greatly improved existing protocols limited by complex sampling procedures and long HPLC analysis times.

A survey of 237 wines from across NYS showed clear differences in the SO₂ binder profiles between different wine types. Red wines were typically higher in α -ketoglutaric acid and galacturonic acid, whereas, white wines were higher in acetaldehyde, pyruvic acid and glucose.

Major regulating factors for acetaldehyde production during alcoholic fermentation were SO₂ addition prior to inoculation, fermentation temperature and grape must type.

With the exception of galacturonic acid, the concentrations of acetaldehyde, pyruvic acid and α -ketoglutaric acid decreased following inoculation with *O. oeni*. Overall bound SO₂ levels were decreased by 22% during MLF and an additional 53% one week later.

During MLF, acetaldehyde bound SO₂ increased mean *O. oeni* lag times in a dose dependant manner. Metabolism of bound SO₂ by *O. oeni* resulted in concomitant increases in free SO₂ concentrations, which never rose about 8.0 mg l⁻¹. Malic acid was depleted by *O. oeni*, despite the presence of acetaldehyde bound SO₂.

Findings suggest that significant reductions in SO₂ binders can be achieved by both yeast and bacterial metabolism during vinification. However, *O. oeni* growth may be limited by the presence of excessive bound SO₂ concentrations. To decrease acetaldehyde levels during cellaring, oxygen ingress should be completely restricted.

BIOGRAPHICAL SKETCH

John Nicholas “Nick” Jackowetz was born in North York, Ontario, Canada on December 17th 1985. He was named after his father and grandfather, though he commonly goes by other titles such as Nick, Potats, Potes, Nickel-Jack and Jacko amongst other aliases not mentionable in polite company. Nick was raised in Brantford, Ontario, Canada, birthplace of Alexander Graham Bell’s telephone and Canadian hockey legend Wayne Gretzky. In 2003 a young, naïve Nick graduated from Assumption college school and enrolled at the University of Guelph in Guelph, Ontario, Canada to study Biomedical Toxicology. While perusing his undergraduate degree, Nick took a summer job in the laboratory of Dr. Ramón Mira de Orduña investigating wine aroma compounds. It was during this time that Nick was first exposed to the scientific study of food and beverage, an experience that would ultimately prove to be life changing. Following his undergraduate graduation in 2007, Nick began his doctoral studies at Cornell University in the renowned department of Food Science in Geneva, NY with Dr. Mira de Orduña, now relocated from Guelph. Nick’s doctoral studies strived to reduce excessive sulfite additions to wine, through the management of SO₂ binding compounds. Upon completion of his dissertation, Nick left his cozy academic cocoon and began his career as a product development specialist for Constellation Wines U.S. in Canandaigua, NY.

This work is dedicated to my parents, John & Anne Jackowetz, my sister Lindsay and my brother Matthew for their unconditional love and support. I have learned so much from each of you over the years and I am extremely lucky to call you all family.

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Sulfur dioxide (SO₂) is an important wine preservative due to its anti-microbial (Carreté et al., 2002), anti-oxidant (Danilewicz, 2003) and anti-enzymatic (Main and Morris, 1991; Wedzicha et al., 1991) functions. However, many consumers view sulfites as unnatural and unhealthy additives based on reports that sulfites are associated with adverse effects from wine consumption (Stolz and Schmid, 2008). While sulfites are safe for most of the population, some individuals, especially asthmatics, do report ill effects from direct sulfite ingestion (Snelten and Schaafsma, 1992; Vally et al., 1999). Accordingly, legal limits for sulfites around the world have become increasingly strict in recent years, especially in the European Union and South Africa. The preservative qualities of SO₂ are greatest when it exists in a free or unbound state in wine. However, the presence of SO₂ binding compounds can limit the potency of SO₂ (Rankine, 1968; Ribéreau-Gayon et al., 1998), leading to increased total SO₂ additions to wine for equivalent preservative action. Hence, an effective strategy for SO₂ reduction in wines is to limit SO₂ binding compounds. Acetaldehyde is quantitatively among the most important SO₂ binding compounds in wine and like SO₂, may also have toxicological implications for consumers (Baan et al., 2007; Lachenmeier and Sohnius, 2008; Matsuda et al., 2006).

This dissertation focuses on the management of SO₂ binding compounds from a microbiological perspective. As such, the impact of yeast and bacterial metabolism on SO₂ binders, especially acetaldehyde, are discussed. The overall goal of this work is to better understand how important SO₂ binding compounds are produced during

vinification so that more SO₂ may exist in a “free” state in wine where it active as a preservative, and not in a bound state, where its preservative efficacy is diminished.

1.2. History of Wine

Wine is an alcoholic beverage and commonly produced from the alcoholic fermentation of the juice of grapes. It has been a staple in the human diet for millennia and was one of the safest liquids to drink historically, due to its high alcohol content and low pH (Pretorius, 2000). Archaeologists have found evidence for the production of a fermented beverage in China in 7000 BC (McGovern et al., 2004). Wine was first mentioned in text from Mesopotamia (present day Iraq and Syria) dating back to 2750 BC (Pretorius, 2000), and was referred to as "liquor of the mountains" due to the suitability of grape vines to the arid, higher elevations as opposed to the humid, low lying valleys. Throughout history, grape cultivation and wine production have spread around the Mediterranean Sea towards Greece (2000 BC), Italy (1000 BC), and later Northern Europe (100 AD) and North America (1500 AD) (Pretorius, 2000).

1.2.1. Louis Pasteur and Enology

Although humans have been making wine since antiquity, the science behind winemaking and fermentation remained poorly understood. In the mid 19th century French scientist Louis Pasteur revolutionized the study of pathogens with his germ theory of disease, proposing that microorganisms are responsible for many human and animal ailments (Bordenave, 2003). Pasteur also applied these principles to the study of food spoilage, and was the first to discover that yeast are responsible for alcoholic fermentation (AF) in grape juice by converting sugars into carbon dioxide and ethanol

(Fleet and Heard, 1993). The study of food microbiology was born from Pasteur's work, although he should also be credited as a pioneer of enology.

1.3. Microbiology of Winemaking

1.3.1. *Saccharomyces cerevisiae*

Enology is the study of wine and winemaking. The concept of making wine is inherently simple, however the art of producing good wine is much more complex. Winemaking begins with obtaining grapes and either fermenting on the skins (red wine) or pressing the grapes and fermenting the juice (white wine). Pasteur discovered that yeast were responsible for the transformation of grape juice into wine. In particular the species *Saccharomyces cerevisiae*, is the predominant yeast during wine fermentations (Walker, 1998). It is hypothesized that as man migrated, they brought vine cuttings and also *S. cerevisiae* in a commensal relationship (Legras et al., 2007). *S. cerevisiae* typically dominate in most fermentations where they are present due to their quick production of, and high tolerance to, ethanol.

1.3.1.1. Alcoholic Fermentation

Under anaerobic conditions *S. cerevisiae* metabolize 1 mole of glucose yielding 2 moles of ethanol, CO₂ and ATP, the cellular energy currency. If this energy output is compared to *S. cerevisiae* cells grown in the presence of oxygen, up to 38 ATP can be produced per mol of glucose (Ribéreau-Gayon et al., 1998). *S. cerevisiae* will also ferment sugars in the presence of oxygen if sugar concentrations are sufficiently high (>9.0 g l⁻¹)(Walker, 1998), an observation called the Crabtree effect (Crabtree, 1928). Why does *S. cerevisiae* choose such an inefficient route for energy conservation? Ultimately, there is an evolutionary advantage to this phenomenon, whereby simple

processing of sugar allows yeast to grow at a faster rate, since they do not have to invest energy into making complex enzyme systems capable of extracting more energy from sugars (Fleet and Heard, 1993). Secondly, the fast growth also coincides with production of large amounts of ethanol, which kill off other organisms competing for same food source, notably bacteria (Thomson et al., 2005).

1.3.1.2. Commercial Strains of *S. cerevisiae*

To ensure dominance of *S. cerevisiae* during commercial, large-scale fermentations, it is common practice to inoculate a strain of *S. cerevisiae* with known characteristics. Desirable yeast traits include: sulfite tolerance, reduced hydrogen low sulfide production, low nitrogen requirements, alcohol tolerance, low foam production, cold and heat tolerance and the formation of desirable aroma compounds. Prior to the 1960's, viable yeast cultures were maintained at the winery throughout the year, and were inoculated into the first grape must during harvest (Fugelsang and Edwards, 2007). However, this process was time consuming, costly, and prone to genetic drift and contamination by other yeast strains and bacteria. In 1963, Red Star Yeast developed the first commercially available active dry yeast (Fugelsang and Edwards, 2007) that could be stored for long periods of time without great loss of yeast viability. For this, yeast are grown in suitable media under high oxygen and low sugar concentrations to avoid both the Pasteur and the Crabtree effect. Upon reaching maximum growth, yeast are harvested, concentrated by filtration and dried with warm air on a conveyor belt (Walker, 1998). Today the use of active dry yeast is ubiquitous in the wine industry with the vast majority of producers inoculating selected strains

(Fugelsang and Edwards, 2007). This also gives winemakers the opportunity to use yeast suitable for a particular grape variety and vintage.

1.3.1.3. Indigenous Non-*Saccharomyces* Yeast

While the direct inoculation of a selected yeast may produce expected results, this strategy may be less popular among winemakers seeking a minimal intervention approach and has the reputation of producing wines that lack body and “complexity” (Ciani et al., 2009). Currently, there is increased interest amongst winemakers to conduct “spontaneous” fermentations, which means that indigenous microbiological flora from the grape or from the winery will ferment the juice into wine. This strategy is thought to produce enhanced aromas (Mendes Ferreira et al., 2001), a fuller, rounder palate and a wine that is reflective of the microbial biodiversity of a given region (Lambrechts and Pretorius, 2000). In response to these concerns, commercial yeast producers have started to offer non-*Saccharomyces* cultures for inoculation to mitigate fermentation risk and provide more reproducible outcomes.

1.3.2. *Oenococcus oeni*

1.3.2.1. Distinguishing Traits and Metabolism of Sugars

In addition to yeast, lactic acid bacteria (LAB) may also impact the final wine product. LAB are responsible for the secondary fermentation commonly encouraged in most red and some white wines, called malolactic fermentation (MLF). During this fermentation, a biological deacidification of wine occurs where L-malic acid is decarboxylated into L-lactic acid, increasing the pH and microbiological stability of the wine (Henick-Kling, 1993). The primary species of LAB responsible for MLF is *Oenococcus oeni*, a Gram positive, non-motile, aerotolerant, catalase and oxidase

negative bacterium of ellipsoidal to spherical shape that occur in pairs or chains (Fugelsang and Edwards, 2007) with an obligatively anaerobic metabolism. *O. oeni* was reclassified from *Leuconostoc oenos* by Dicks et al. (1995) because of major physiological differences to other LAB including growth at low pH and tolerance to high ethanol concentrations. *O. oeni* are classified as heterofermentative because they lack the aldolase enzyme used during glycolysis and consequently ferment sugars via the phosphoketolase pathway (Fugelsang and Edwards, 2007). Under anaerobic winemaking conditions *O. oeni* will metabolize 1 mole of glucose to produce 1 mole of lactate, CO₂, ethanol or acetic acid and 1 mole of ATP. Oxygenation of wine during MLF will increase the concentration electron accepting molecules and will result in the production of acetic acid by *O. oeni* as a means of

1.3.2.2. Malolactic Fermentation

Metabolism of glucose is a traditional mechanism of energy assimilation for many living organisms, however LAB have developed another unique process of obtaining ATP, while using the harsh environment of wine to their advantage (Cox and Henick-Kling, 1990). LAB are able to produce ATP through malate metabolism using principles devised in the chemiosmotic theory, whereby energy is harnessed through the use of electrochemical gradients across a membrane (Mitchell, 1961). Decarboxylation of malate to lactate will consume a proton inside the LAB cell and increase the internal pH. This proton differential between the inside and outside of the cell results in a proton gradient that can be used to drive a membrane bound ATPase, producing ATP (Olsen et al., 1991; Poolman et al., 1991).

1.3.2.3. Aroma Modification and Secondary Metabolites

Besides their role in biological deacidification, LAB are also important during winemaking because of their impact on wine aroma. Under favorable conditions, *O. oeni* produce few off-odors and have been found to decrease vegetative aromas in wine while increasing concentrations of varietal aroma compounds from precursors due to their glycosidase activity (Bartowsky and Henschke, 1995). However, under certain conditions (e.g. oxidative) LAB can increase levels of diacetyl and acetic acid which can be detrimental to overall wine quality (Bartowsky and Henschke, 2000). Additionally, certain strains of LAB can produce polysaccharides leading to increased wine viscosity and “ropiness” (Lonvaud-Funel and Joyeux, 1988). Biogenic amine and ethyl carbamate precursor production is another concern from LAB metabolism and have been implicated as the casual agents for many adverse health reactions associated from wine consumption (Lonvaud-Funel, 1999; Silla Santos, 1996; Stratton et al., 1991). Hence, winemaking strategies that limit these potential adverse health agents are important during quality wine production.

1.4. Sulfur Dioxide

1.4.1. History and Use

SO₂ is perhaps the best known preservative in wine, which many consumers associate with negative health effects following wine consumption (Smith, 2002; Tollefson, 1988). Yet, SO₂ has been used for centuries and a disinfectant and antibacterial. The ancient Greeks used SO₂ to fumigate their homes and the Romans and Egyptians used it to cleanse wine receptacles (Lester, 1995). Today, SO₂ is widely used due to its anti-microbial (Carreté et al., 2002), anti-enzymatic (Main and Morris, 1991; Wedzicha et al., 1991) and anti-oxidant (Danilewicz, 2003) properties. SO₂ is added to many foods

including, seafood, potato preparations, fruit drinks, baked goods and dried fruits to name a few (Doyle and Beuchat, 2007; Yang and Purchase, 1985).

1.4.2. Mechanisms of Action

1.4.2.1. Anti-Microbial Activity

When dissolved in water SO_2 will form an acid that will dissociate in relation to the pH of the medium, based on the Henderson-Hasselbalch equation (Figure 1.1). For instance, at wine pH (3.0-4.0) the dominant species is the bisulfite ion, with a small contribution of molecular SO_2 , which is thought to be the most active SO_2 species (Ribéreau-Gayon et al., 1998). Molecular SO_2 can act as a bactericidal agent by diffusing through cell membranes and inhibiting cellular enzymes through cleavage of disulfide bonds (McWeeny, 1979), ultimately obstructing ATP production (Maier et al., 1986; Schimz, 1980; Schimz and Holzer, 1979). SO_2 is also known to deaminate cytosine to uracil (Fugelsang and Edwards, 2007), causing lethal mutations, it can bind to FADH_2 and NADH co-factors negatively effecting cellular redox balance (Fugelsang and Edwards, 2007) it destroys thiamine (Studdert and Labuc, 1991), a necessary vitamin for yeast and bacteria, and reacts with ATPase decreasing available ATP pools (Carreté et al., 2002).

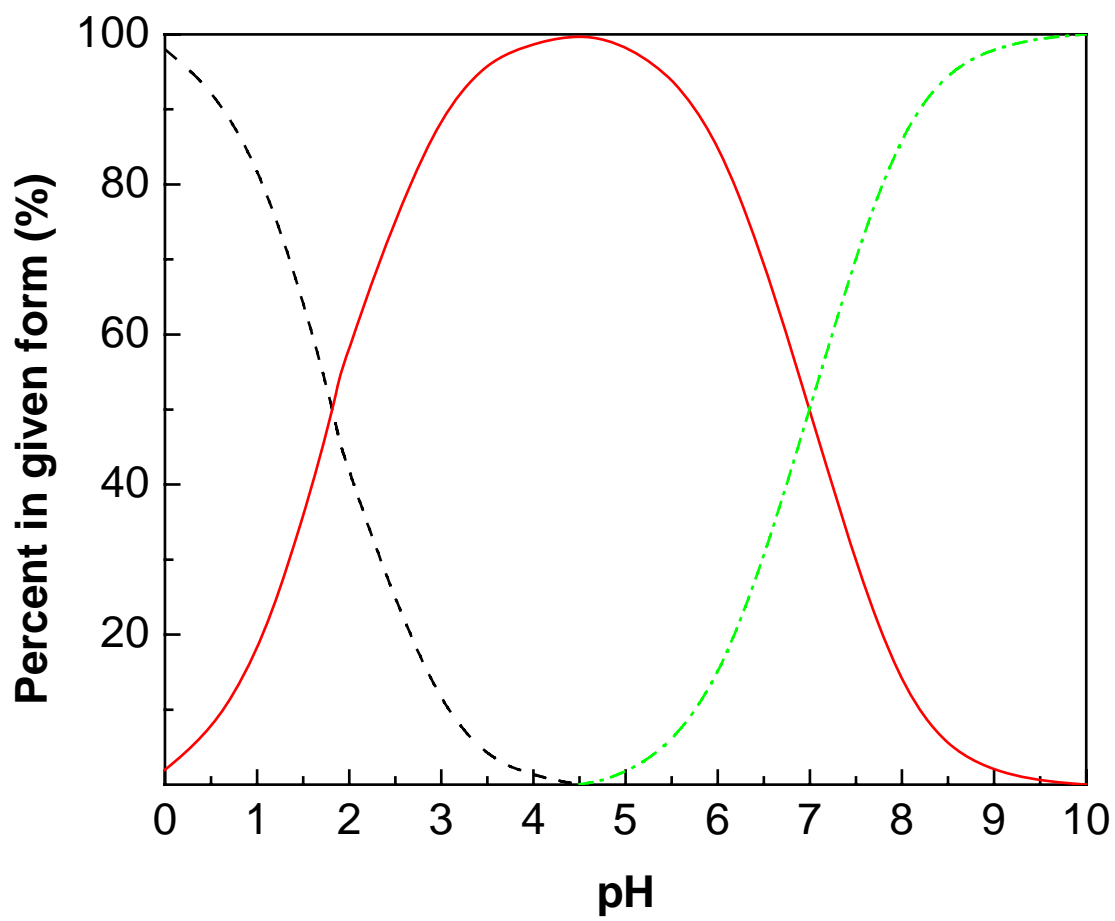


Figure 1.1 Speciation of SO_2 over a wide pH range. SO_2 (---), HSO_3^- (—), SO_3^{2-} (-.-).

1.4.2.2. Anti-Enzymatic Activity

For the preservation of fruits and vegetables, SO₂ is valued for its antienzymatic ability, especially its inhibition of the browning enzyme, polyphenol oxidase (Iyengar and McEvily, 1992). Sulfites are thought to inhibit non-enzymatic browning, as well, by acting as a reducing agent that reacts with the ortho-quinones and converts them to colorless diphenols (Danilewicz, 2003). SO₂ is especially important in white wines to help maintain a commercially acceptable color, and limit unnecessary darkening of wine from chemical oxidation.

1.4.2.3. Anti-Oxidant Activity

Control of oxidation reactions during wine aging and storage is imperative for the production of quality wine. Danielwicz (2007) demonstrated that wine oxidation is a complex process involving interactions between polyphenolics, transition metals and oxygen resulting in reactive species and oxidation reactions (Danilewicz, 2007). At the center of this oxidative mechanism are iron and copper ions that act as catalysts and transfer electrons to polyphenols, which then react with oxygen leading to peroxide formation and hydroxyl radicals. Peroxide then reacts rapidly via the Fenton reaction (Elias and Waterhouse, 2010) producing radical compounds, which may adversely affect the color and aroma of wine. The strong nucleophilic properties of SO₂ prevent this oxidation cascade by reducing ortho-quinones back to diphenols and also reacting directly with peroxide preventing radical formation (Danilewicz, 2007; Elias and Waterhouse, 2010). Hence, the use of SO₂ during winemaking is very important for quality wine production. Vinifications where SO₂ was not added yielded wines that

were generally less accepted by a tasting panel than those wines with SO₂ added (Ough and Cromwell, 1987).

1.4.3. Sulfites and Health

However, the use of SO₂ in winemaking is still viewed by many consumers as unnatural and unhealthy (Stolz and Schmid, 2008). These apprehensions about SO₂ utilization may stem from historical excessive use of sulfites for vegetable preservation at grocery stores and restaurants (Martin et al., 1986; Settippane, 1987) and also sulfite warning labels on most wine bottles. Sulfite pathogenesis is a complex process known to occur as intolerances (non-allergic) and more rarely by anaphylaxis (allergic) mediated mechanisms (Wüthrich, 1993). Most adverse (non-allergic) sulfite reactions occur via a neural reflex action involving irritant receptors in the nose. Stimulation of these irritant receptors causes a cholinergic efferent response resulting in immediate flushing, acute bronchospasm and hypotension in sensitive subjects (Settipane, 1987). In non-sensitive individuals, endogenous sulfite is maintained at a very low levels and is metabolized by sulfite oxidase producing sulfate, which is excreted in the urine (Lester, 1995). A decrease in sulfite oxidase activity may explain prevalence of sulfite sensitivity in some individuals (Lester, 1995), and it is estimated that 1% of the U.S. population is sulfite sensitive (Papazian, 1996). In particular, asthmatics appear to be especially susceptible, with estimates that up to 5% may risk adverse reactions upon sulfite exposure (Snelten and Schaafsma, 1992; Vally et al., 1999). Incidences of sulfite sensitivity were found to be higher in women compared with men, and the mean age of sensitive individuals was 40 years old. (Lester, 1995). In a review by Vally & Thompson (2003) sulfite sensitivity in asthmatics was

heightened by other parameters that increase asthmatic stress such as cigarette smoke, pollen allergy, alcohol intake and biogenic amine consumption. Therefore, the negative health effects observed during from sulfite consumption may be difficult to quantify in a reproducible manner because of synergistic effects with other confounding factors.

1.4.4. Sulfite Regulations

Because of these health concerns, regulations have been implemented to prevent excessive consumption of sulfites in the diet. In 1985, The FDA contracted the Federation of American Societies for Experimental Biology (FASEB) to examine the link between sulfites and the reported health claims. The FASEB concluded that sulfites are safe for most people, but could pose a hazard of unpredictable severity to asthmatics and others who are sensitive to them (Papazian, 1996). Based on this report, sulfites were prohibited from use in fruit and vegetable preparations for maintenance of color and crispness, such as in salad bars or fresh produce in supermarkets (Tollefson, 1988). This report also lead to the mandatory labeling of food products containing more than 10 mg l^{-1} of sulfites on food labels (27 CFR 4.32(e)). However, North American regulations regarding SO_2 in wine are currently less strict than in the European Union and other wine producing countries (Table 1.1). These regulatory discrepancies may also limit the trade of wines with excessive SO_2 concentrations into certain jurisdictions affecting overall winery profitability.

Table 1.1 Legal limits for total SO₂ in major winemaking nations (in mg l⁻¹)¹⁾

Country	Wine type, RS	Limit	Legal Reference/Description
USA	All	350	27 CFR 4.22(b)(1)
AUS	<35 g/l sugars	250	ANZFSC 4.5.1: Clause 5(5)(a)
	>35 g/l sugars	300	
NZ	<35 g/l sugars	250 ²⁾	
	>35 g/l sugars	400 ²⁾	
EU	white/rosé, <5 g/l	200	EC No 606/2009, Annex I B
	red, <5 g/l sugars	150	
	white/rosé, >5 g/l	250	
	red, >5 g/l sugars	200	
	specific wines	300	E.g.: Spätlese (can be dry), Bordeaux Sup., Côtes de Bordeaux, C. de Bergerac, Navarra, Penedès, several French VdP and Hungarian and some Greek sweets
	specific wines	350	E.g.: Auslese (can be dry), sweet wines from Romania, Czech Rep., Slovakia and Slovenia
	specific wines	400	E.g.: Beerenauslese, TBA, Eiswein, French sweet wines such Sauternes, Barsac, etc., sweet Greek with >45 g/l sugars, sweet Eastern European wines
CAN	All	350 ³⁾	Canadian Food & Drug Reg. B.02.100
India	All	450	Prevention of Food Adulteration Act & Rules, Appendix C, Table 3
Japan	All (>1% abv)	350 ²⁾	Japan's Specifications and Standards for Food Additives
RSA	white, <5 g/l sugars	160	Liquor Products Act 60 of 1989 Regulations Regulation 32 (Table 8)
	reds, <5 g/l sugars	150	
	All, >5 g/l sugars	200	
	specific wines	300	E.g.: noble late harvest and naturally dried

¹⁾ Information retrieved from FIVS-Abridge database (www.fivs-abridge.com)

²⁾ unit is mg/kg

³⁾ Canada prescribes a maximum of 70 mg/L free or 350 mg/L combined SO₂

1.4.5. Consumer Interest in Low Sulfite Foods

Minimally processed foods and wines, with little or no sulfites have become increasingly popular with consumers (Azabagaoglu et al., 2007; Koehr, 2006), especially young people (Magnusson et al., 2001). Many customers are concerned about the health implications from chemical additives to wines (Smith, 2002). In one study, wine consumers expressed their willingness to pay a premium of 16.5% for organically produced products (Molla-Bauza et al., 2005). Sales of certified organic foods in the U.S. have increased from \$78 million in 1980 to approximately \$6 billion in 2000, with an average annual increase of 24% during the 1990s (Hughner et al., 2007). The organic wine sector has also followed this trend. In 2005, the world organic wine market grew by 10 to 15% (Richter and Padel, 2007). For a wine to be labeled organic in the U.S. it must abide by a SO₂ limit of 10 mg l⁻¹, which may only come from yeast production and not exogenous additions. Conversely, wine labeled “produced from organically grown grapes” must adhere to a limit of 100 mg l⁻¹ of total SO₂ (7 CFR part 205).

1.5. Sulfur Dioxide Binding Compounds

The nucleophilic properties of SO₂ favors its reaction with electrophilic compounds in wine, notably carbonyls and ketones (Ribéreau-Gayon et al., 1998). However, bound forms of SO₂ are thought to have decreased preservative activity compared with free or “active” SO₂ (Rankine, 1968; Ribéreau-Gayon et al., 1998). Hence, wines with high concentrations of SO₂ binding compounds typically require increased SO₂ additions for equivalent product stabilization.

SO₂ binders can arise from grape tissue, from microorganisms during fermentation, and from chemical reactions in wine (Figure 1.2). Of all the relevant SO₂ binding compounds in wine, acetaldehyde binds to SO₂ with strongest affinity, explaining the low dissociation constant of its sulfonate ($K_d = 1.5 \times 10^{-6}$) (Burroughs and Sparks, 1973). The following sections will discuss SO₂ binders commonly found in wine in decreasing order of sulfonate bond strength.

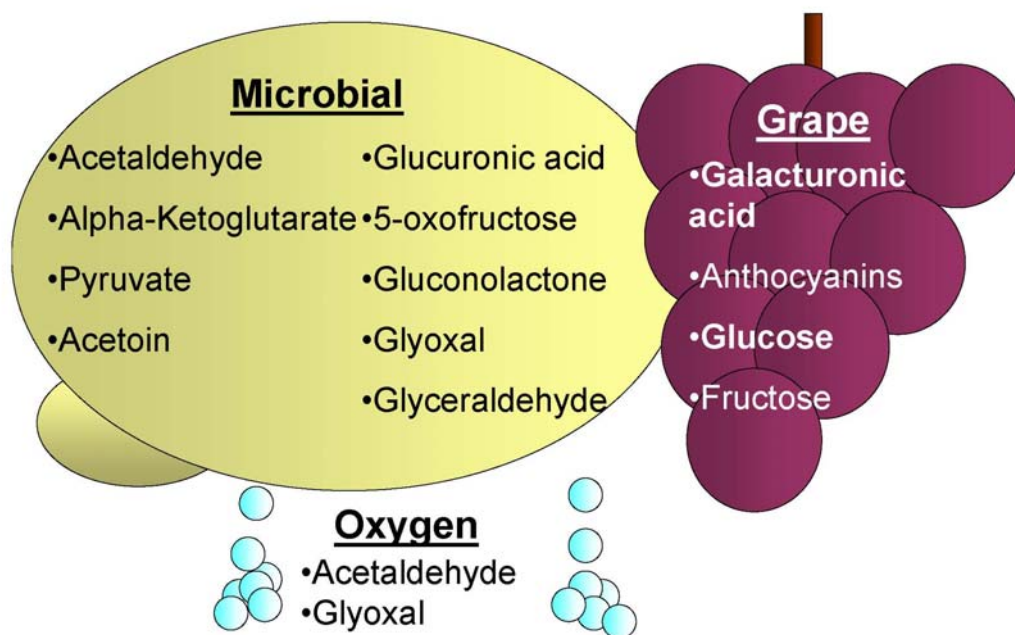


Figure 1.2 Source of SO₂ binding compounds during winemaking operations

1.5.1. Acetaldehyde

Acetaldehyde is a reactive, low molecular weight, flavor active compound, important for color perception and found in a variety of foods and beverages such as cheese (Weerkamp et al., 1996), yogurt (Bottazzi et al., 1973), beer (Margalith, 1981) and wine (Bottazzi et al., 1973; Dittrich and Barth, 1984; Margalith, 1981; Weerkamp et al., 1996). High acetaldehyde concentrations impart aromatic attributes reminiscent of green apples, fresh cut grass and walnuts (Margalith, 1981). These sensory descriptors are typical of wines such as Sherry or Vin Jaune (Cullere et al., 2007), which usually contain higher levels of free or unbound acetaldehyde, but are undesired in most other table wines. During alcoholic fermentation, acetaldehyde will arise during yeast metabolism of hexose sugars when pyruvate is decarboxylated via pyruvate decarboxylase (Venturin et al., 1995; Wang et al., 2006). Some studies have suggested that acetaldehyde reaches a peak value during the yeast exponential growth phase and is then partly reutilized throughout stationary phase (Osborne, 2006; Weeks, 1969). Acetaldehyde can also result from chemical oxidation of ethanol when wine is exposed to oxygen, post-AF (Danilewicz et al., 2003).

Acetaldehyde is also produced in the human body following consumption of foods or beverages containing ethanol. Upon ingestion, ethanol is quickly absorbed and transported to the liver where it oxidized into acetaldehyde by the alcohol dehydrogenase enzyme (Sidhu and Blair, 1975). Subsequent metabolism by aldehyde dehydrogenase (ALDH) further oxidizes acetaldehyde into acetate.

However, there are some individuals who cannot efficiently degrade acetaldehyde *in vivo*, due to low ALDH activities, resulting in high circulating concentrations of

acetaldehyde and manifestation of adverse health effects (Baan et al., 2007). Perhaps the most obvious sign of acetaldehyde toxicity is facial flushing. More serious effects from acetaldehyde were shown by Yokoyama et al. (1998) who found that those individuals with low AIDH activity were at higher risk (odds ratios) for oropharyngolaryngeal (11.14), esophageal (12.50), stomach (3.49), colon (3.35), lung (8.20) and esophageal cancer concomitant with oropharyngolaryngeal and/or stomach cancer (54.20). These health issues may result from the non-specific binding of acetaldehyde with enzymes and DNA, resulting in decreased cellular efficiency and possible mutations (Baan et al., 2007; Matsuda et al., 2006).

The possible effects of direct acetaldehyde consumption have been evaluated by Lachenmeier, Kanteres & Rehm (2009). They concluded that acetaldehyde contained in alcoholic beverages may increase the risks of upper gastrointestinal tract cancers, simply from direct exposure through alcoholic beverage consumption. A large survey by the same authors found that acetaldehyde concentrations in saliva were typically highest in fortified wines followed by spirits and table wines (Lachenmeier and Sohnius, 2008).

Despite the current scientific literature suggesting otherwise, acetaldehyde does have GRAS (generally regarded as safe) status by the US FDA (21 CFR 182.60) and is included in the European Union's register of flavoring substances. Acetaldehyde may also be directly added to wine up to 300 mg l⁻¹ for the purposes of color stabilization according to US regulations (27 CFR 24.246). However, due to its reactivity and potential carcinogenicity, it has been recommended that acetaldehyde levels in

alcoholic beverages be as low as possible, while restricting its use as a food flavor additive (Lachenmeier et al., 2009).

1.5.2. Pyruvate

Pyruvate is another important SO₂ binding compound found in wine. Although it does not bind SO₂ as strongly as acetaldehyde, pyruvate can bind significant amounts of SO₂ explaining the relatively low dissociation constant of its sulfonate ($K_d = 2.0 \times 10^{-4}$) (Ribéreau-Gayon et al., 1998). Pyruvate is produced at high levels during the early stages of AF when pyruvate decarboxylase enzyme is inhibited by aerobically cultured yeast (Fleet, 1993). LAB can also produce pyruvate from several sources including sugars, citric and amino acids (Liu, 2003). Biological degradation occurs via both yeast and LAB (Swiegers et al., 2005). Yeast primarily decarboxylate pyruvate into acetaldehyde (Fleet, 1993). Whereas, LAB mainly produce lactic acid from pyruvate, though other products such as acetate, formate, ethanol, acetaldehyde, diacetyl, acetoin and 2,3-butanediol are also formed (Liu, 2003; Zaunmuller et al., 2006).

1.5.3. α -Ketoglutaric acid

α -Ketoglutaric acid is an intermediate compound during amino acid metabolism in yeast and bacterial cells. Yeast can produce and metabolize α -ketoglutaric acid during AF (Moreno-Arribas and Carmen Polo, 2009). Some strains of heterofermentative LAB including *O. oeni* degrade α -ketoglutaric acid, producing γ -butyrolactone (Gambaro et al., 2001). This reaction pathway was found to be most active when electron acceptors such as fructose, citrate or pyruvic acid were also present in wine (Vermeulen et al., 2006).

1.5.4. Galacturonic Acid

Galacturonic acid is the monomer of pectin, found predominantly in the skin of the grape (Lopez-Tamames et al., 1996). Pectin content increases steadily throughout grape ripening and can reach levels up to 1 g l^{-1} in some cases (Fugelsang and Edwards, 2007). Upon maturation of the grape, endogenous pectinase levels increase resulting in a softening of the berry and liberating galacturonic acid (Winkler et al., 1974). Galacturonic acid can also be liberated by the actions of exogenous pectinase addition used by winemakers to increase juice yields (Ugliano, 2009). Galacturonic acid levels in grapes are dependant on cultivar, humidity levels and annual rain fall in the growing area (Lopez-Tamames et al., 1996). Its concentration is also influenced by the actions of the grape spoilage mold *Botrytis cinerea*, who also produce the pectinase enzyme (Francioli et al., 1999).

1.5.5. Acetoin

Acetoin may also bind SO_2 to a limited degree and is produced from pyruvate by both yeast and LAB during fermentation (Guymon and Crowell, 1965). During MLF, LAB metabolize citric acid and sugars producing acetoin (Bartowsky and Henschke, 1995). In Sherry wines, acetoin can reach levels up to 350 mg l^{-1} (Cortes et al., 1998), though in most table wines its concentrations ranges from 5 to 20 mg l^{-1} (Bartowsky and Henschke, 1995).

1.5.6. Glucose

Besides water, glucose and fructose comprise are some of the most abundant organic compounds found in grape must. Glucose is an important molecule for growth and energy production of most living organisms and also has the ability to bind with SO_2 ,

albeit to slight degree (Ribéreau-Gayon et al., 1998). However, its high concentrations in must and wine compensate for its weak reactions with sulfites, especially in sweet or dessert wines (Jarvis and Lea, 2000).

1.6. Dissertation Objectives

This introduction has presented previous research relevant to the management of SO₂ binding compounds throughout vinification, with special emphasis of microbiological control techniques. Recently, there has been great consumer interest into organic or minimally processed wines stemming from perceived health concerns of preservatives, like SO₂, traditionally used during wine processing. To meet these market demands the production of wines with low sulfite levels has increased, yet this restriction presents challenges for the production of quality wines. The presence of SO₂ binding compounds in wine can greatly reduce the efficacy of SO₂ and necessitate increased addition of SO₂ to wines. These additions may restrict the international trade of wine due to strict sulfite regulations in some jurisdictions. Accordingly, the aim of this dissertation project was to investigate important SO₂ binding compounds throughout vinification and develop strategies for their control to increase the preservative efficacy of SO₂ and limit total additions to wine.

The first objective of this study was to develop a comprehensive, robust method for the analysis of important SO₂ binding compounds in wine. This method derivatized wine SO₂ binders to 2,4-dinitrophenylhydrazine (DNPH) and then separated derivatives on an ultra high performance liquid chromatography (UHPLC) system. Special efforts were made to limit sample oxidation and interferences from other wine matrix compounds (Chapter 2).

A survey of 237 New York State wines was then conducted to gain a better understanding of common concentrations and distributions of SO₂ binding compounds across different wine types. Extension efforts with local winemakers were also explored to help isolate critical control points for acetaldehyde production during commercial vinifications.

(Chapter 3).

Chapter 4 investigated the production of acetaldehyde throughout alcoholic fermentation to better identify its regulating factors. Careful monitoring of oxygen using a non-invasive fluorescence quenching probe during fermentation helped ensure that all measured acetaldehyde concentrations were from yeast and not due to chemical oxidation of ethanol from oxygen ingress.

The possible metabolism of SO₂ binding compounds was observed during and after malolactic fermentation in MRS media. Estimated bound SO₂ concentrations were calculated to determine the optimal time for wine stabilization (Chapter 5).

Finally, the effects of pure acetaldehyde bound SO₂ were observed on the growth and metabolism of *Oenococcus oeni* during malolactic fermentation. Strategies for successful malolactic fermentation under difficult conditions will be discussed (Chapter 6).

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CHAPTER 2: IMPROVED SAMPLE PREPARATION AND RAPID UHPLC ANALYSIS OF SO₂ BINDING CARBONYLS IN WINE BY DERIVATIZATION TO 2,4-DINITROPHENYLHYDRAZINE

2.1. Abstract

Sulfur dioxide (SO₂) is essential for the preservation of wines. The presence of SO₂ binding compounds in musts and wines may limit sulfite efficacy leading to higher total SO₂ additions, which may exceed SO₂ limits permitted by law and pose health risks for sensitive individuals. An improved method for the quantification of significant wine SO₂ binding compounds is presented that applies a novel sample treatment approach and rapid UHPLC separation.

Glucose, galacturonic acid, alpha-ketoglutarate, pyruvate, acetoin and acetaldehyde were derivatized with 2,4-dinitrophenylhydrazine and separated using a solid core C18 phase by ultra high performance liquid chromatography. Addition of EDTA to samples prevented *de novo* acetaldehyde formation from ethanol oxidation. Optimized derivatization duration enhanced reproducibility and allowed for glucose and galacturonic acid quantification. High glucose residues were found to interfere with the recovery of other SO₂ binders, but practical SO₂ concentrations and red wine pigments did not affect derivatization efficiency. The calibration range, method accuracy, precision and limits of detection were found to be satisfactory for routine analysis of SO₂ binders in wines.

The current method represents a significant improvement in the comprehensive analysis of SO₂ binding wine carbonyls. It allows for the quantification of major SO₂ binders at practical analyte concentrations, and uses a simple sample treatment method that prevents treatment artifacts. Equipment utilization could be reduced by rapid LC

separation while maintaining analytical performance parameters. The improved method will be a valuable addition for the analysis of total SO₂ binder pools in enological samples.

2.2. Introduction

Sulfur dioxide (SO₂) is an important preservative in wines and other food matrices. It is inexpensive, effective against a broad spectrum of spoilage microorganisms at low levels (Hood, 1983), a potent anti-oxidant (Danilewicz, 2003) and has anti-enzymatic activities (Wedzicha et al., 1991). Several wine compounds with carbonyl or keto groups are known to form covalent adducts (sulfonates) with SO₂.

Binding to such compounds may reduce the preservative activity of SO₂ since it has been shown to be most effective in its free or unbound state, where it is able to diffuse into cells and inhibit cellular enzymes through cleavage of disulfide bonds ultimately obstructing ATP production in susceptible microorganisms (Maier et al., 1986; Schimz, 1980). Hence, large concentrations of SO₂ binding compounds typically require increased additions of SO₂ for equivalent preservative action. Table 2.1 lists significant SO₂ binding compounds found in wine with the dissociation constants (K_d) of their respective SO₂ adducts. Because of the low dissociation constant, acetaldehyde forms particularly stable adducts with SO₂.

Table 2.1 SO₂ binding compounds and respective dissociation constants (K_d) for SO₂ adducts summarized from scientific literature.

SO ₂ Binding Compound	SO ₂ Adduct Dissociation Constant (K _d)	Reference
Glucose	6.4x10 ⁻¹ , 9.0x10 ⁻¹	(Ribéreau-Gayon et al., 1998a; Würdig and Woller, 1989)
Acetoin	8.0x10 ⁻²	(Blouin, 1966)
Galacturonic Acid	1.6x10 ⁻² , 1.8x10 ⁻² , 2.0x10 ⁻²	(Burroughs and Sparks, 1973; Ribéreau-Gayon et al., 1998a; Würdig and Woller, 1989)
Alpha-ketoglutarate	4.9x10 ⁻⁴ , 5.6x10 ⁻⁴ , 6.6x10 ⁻⁴	(Burroughs and Sparks, 1973; Ribéreau-Gayon et al., 1998a; Würdig and Woller, 1989)
Pyruvate	1.4x10 ⁻⁴ , 1.6x10 ⁻⁴ , 2.0x10 ⁻⁴	(Burroughs and Sparks, 1973; Ribéreau-Gayon et al., 1998a; Würdig and Woller, 1989)
Acetaldehyde	1.5x10 ⁻⁶ , 2.4x10 ⁻⁵	(Burroughs and Sparks, 1973; Ribéreau-Gayon et al., 1998a; Würdig and Woller, 1989)

The US FDA estimates that as many as 1% of the American population show an increased degree of sensitivity to sulfites (Papazian, 1996). In particular, asthmatics seem to be especially sensitive to sulfites with estimates that as many as 5% of asthmatics may risk adverse reactions upon SO₂ ingestion (Snelten and Schaafsma, 1992; Vally et al., 1999). Canada and the USA allow a maximum of 350 mg l⁻¹ of total SO₂ regardless of wine style. In New Zealand and Australia, dry wines may not exceed 250 mg l⁻¹, and a limit of 400/300 mg l⁻¹ has been set for wines with >35 g l⁻¹ of residual sugar, respectively (Anonymous, 2011). Europe has a large number of specific limits for sweet specialty wines from the various Appellations. However, the maximum legal limits for all dry red and white wines was recently lowered to 150 and 200 mg l⁻¹, respectively (Anonymous, 2011).

Because of their relevance for wine stabilization, the chemical and biological formation and degradation of SO₂ binding compounds in wine has been at the center of recent studies (Cheraiti et al., 2010; Jackowetz et al., 2011; Osborne et al., 2006). Analysis of individual SO₂ binding compounds using enzymatic methods (Bergmeyer, 1974) is possible, and chemical methods have been applied to the analysis of acetoin (Castro Vazquez et al., 2006) and galacturonic acid (Kintner and Buren, 1982), but applying such methods to survey major SO₂ binding compounds simultaneously is cumbersome and time consuming. The derivatization of carbonyl and keto compounds with 2,4-dinitrophenylhydrazine (DNPH) has been described early on (Brady and Elsmie, 1926), and its application in automated LC systems with spectrophotometric detection has also been presented (de Azevedo et al., 2009; Elias et al., 2008; Lea et al., 2000). Yet, the application of these methods to enological studies is limited

because of their dynamic range, the number of possible analytes, the sample preparation method or its susceptibility towards *de novo* formation of acetaldehyde from ethanol oxidation.

The current work presents and validates an improved method for the quantification of SO₂ binding wine carbonyls using DNPH derivatization and rapid LC analysis. In order to reduce analytical run time, an ultra high pressure LC system (UHPLC) combined with a small particle size column with solid core technology were utilized. Sample preparation was simplified by eliminating the need for anaerobic sample handling required in other methods. The derivatization kinetics between SO₂ binding compounds and DNPH were thoroughly investigated under different conditions to identify potential interferences, ensure method reproducibility and robustness.

2.3. Materials and Methods

2.3.1. Chemicals and preparation of reagents

All chemicals were of all analytical grade and obtained from Thermo Fisher Scientific (Pittsburgh, PA). DNPH had a purity of 97% (as dry weight) and contained 30-40% water to reduce the explosion hazard. A model wine was used as a solvent for calibration standards and contained 6 g l⁻¹ of tartaric acid and 12% ethanol (v/v), and was adjusted to pH 3.6 with sodium hydroxide. ASTM Class I water was prepared using a water purification system (Arium 611UV, Sartorius, Germany) and used throughout the study.

The derivatizing reagent was prepared by dissolving DNPH in acetonitrile adjusted to pH 1.5 with perchloric acid to obtain an 11 mM DNPH solution. For sample pre-treatments, an aqueous solution of 86 mM EDTA in 1 M sodium hydroxide was

prepared and degassed (Aquasonic Model 150D, VWR International, West Chester, PA) for 20 minutes. Solutions were stored up to one week at 4°C.

2.3.2. Derivatization procedure

Derivatizations were conducted in 2.0 ml glass HPLC vials with Teflon caps (National Scientific, Rockwood, TN). For sulfonate hydrolysis, 200 µl of sodium hydroxide/EDTA solution were added to the vial, followed by 100 µl of sample wine. After mixing, the vials were capped. After 10 minutes, 200 µl of 1M perchloric acid were added followed by 800 µl of the derivatizing reagent. After mixing, the solution was allowed to react for exactly 30 hours at $30.0 \pm 0.1^\circ\text{C}$ and then promptly cooled to 4°C until analysis. Derivatized samples thus prepared were stable for up to five days.

2.3.3. Analysis

As a control for the development of the sample pre-treatment method, acetaldehyde (SO₂ bound and unbound) was measured enzymatically using a commercial test kit (Megazyme, Ireland).

An ultra high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AD XR pumping unit, a DGU-20A₃ degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven, and a SPD-20A UV/VIS detector were used for separation and analysis of DNPH derivatized wine carbonyls. Data acquisition and analysis was performed with the LCSolution software (1.23). Solvent A consisted of water acidified to $\text{pH } 2.50 \pm 0.01$ using perchloric acid. Solvent B was HPLC grade acetonitrile. All solvents were filtered prior to utilization (0.22 µm, nylon, Millipore, Ireland). Samples were held at 4°C in the autosampler and 5.0 µl of sample injected directly. Separation occurred on a Phenomenex Kinetex C18 stationary phase (100 x

3.0 mm) with 2.6 μm particle size held at 37°C with a flow rate of 0.75 ml min⁻¹. The analytes were quantified at 365 nm using external calibration standards with linear regression analysis. The separation gradient is shown in Table 2.2. The alternative HPLC method for validation purposes was carried out on the same system and under identical conditions, but with a Shimadzu Shimpak XR-ODS stationary phase with 2.2 μm particle size (100 x 3.0 mm).

Table 2.2 Gradient program for the chromatographic separation of DNPH derivatized SO₂ binding compounds using a Phenomenex Kinetex C18 column (100 x 3.0 mm).

Time (min)	Solvent A	Solvent B
0.00	85	15
4.25	82	18
4.75	75	25
6.50	62	38
12.00	40	60
12.50	10	90
13.25	85	15

2.3.4. Statistical analysis

Student's t-tests were conducted with JMP 7.0 (SAS, North Carolina) to determine statistical significance between sample populations at the 0.05 confidence level.

2.4. Results

An exemplary chromatogram displaying mixed calibration standards at various concentrations is shown in Figure 2.1. Preliminary tests with citric acid and EDTA applied at various concentrations led to the utilization of EDTA at 86 mM for oxidation control in further analyses. To test the ability of EDTA to limit the *de novo* formation of acetaldehyde during the 30-hour derivatization procedure, its application in open and closed vials was compared with rapid enzymatic quantification of acetaldehyde in a Riesling wine. The results demonstrated that there was no statistically significant difference between samples with added EDTA analyzed by HPLC and the enzymatic method regardless of atmospheric exposure. In contrast, omission of EDTA led to the observation of a 2.5 fold increase of acetaldehyde both in closed and open vials (Table 2.3).

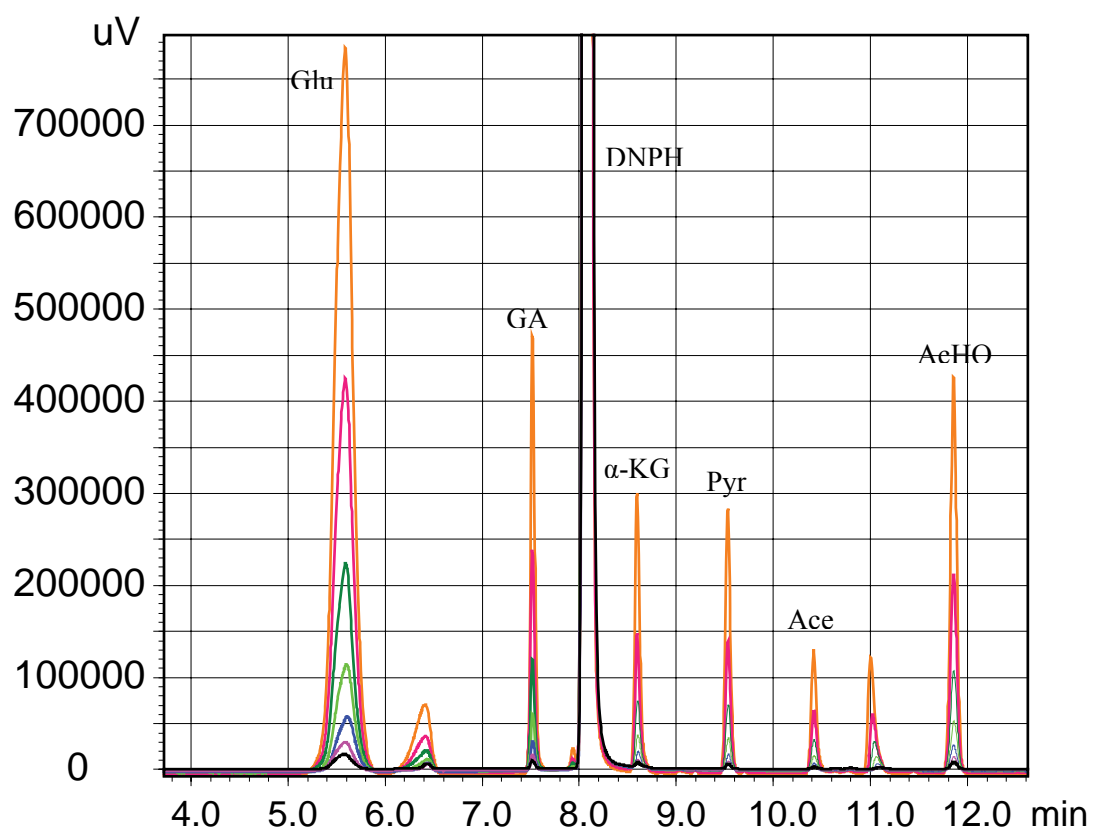


Figure 2.1 Chromatogram of mixed calibration standards in wine using a solid core C18 stationary phase (100 x 4.6 mm, Phenomenex Kinetex). Colors represent different concentrations of standards, low to high (black to orange). SO₂ binding compounds are eluted in the following order, (Glu) glucose; (GA) galacturonic acid; (α -KG) alpha-ketoglutarate; (Pyr) pyruvate; (Ace) acetoin; (AcHO) acetaldehyde. DNPH peak represents unreacted derivatizing reagent and peaks at 6.4 and 11.0 minutes are artifacts from calibration standards.

Table 2.3 Effect of different sample preparation techniques on acetaldehyde concentrations determined in Riesling samples as analyzed by UHPLC following DNPH derivatization.

Treatment	Acetaldehyde (mg l ⁻¹)	
	EDTA	No EDTA
Closed vial	14.1±0.2 ^a	35.5±1.1 ^b
Open vial	13.7±0.1 ^a	33.9±0.9 ^b
Enzymatic control	14.6±0.6 ^a	

Different letters indicate statistically significant differences at <0.01 confidence level.

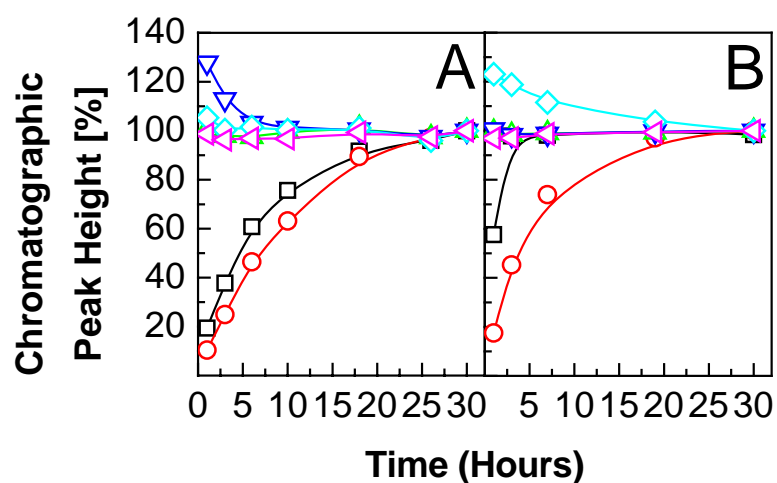


Figure 2.2 Effect of incubation time and temperature on derivatization efficiency as displayed by relative chromatographic peak height. 100% defined as the maximum peak height obtained for individual SO₂ binding compounds after 30 hours. Incubation temperature (A) 30°C and (B) 50°C. (□) glucose (6.25g l⁻¹), (○) galacturonic acid (450 mg l⁻¹), (△) alpha-ketoglutarate (100 mg l⁻¹), (▽) pyruvate (100 mg l⁻¹), (◇) acetoin (50 mg l⁻¹) and (◁) acetaldehyde (60 mg l⁻¹).

The effect of incubation duration (up to 30 hours) and temperature (30°C and 50°C) on the derivatization efficiency of carbonyls with DNPH was evaluated with standards in a buffer. Figure 2.2 shows that a stable equilibrium was established for all selected SO₂ binders and DNPH after 30 hours at both 30°C and 50°C, with galacturonic acid and glucose being the slowest reactants. Increasing the incubation temperature to 50°C accelerated the derivatization of galacturonic acid and glucose. However, verification of higher incubation temperatures with samples of several alcoholic beverages showed that oxidative reactions increased despite EDTA addition. Specifically, at 50°C incubation temperature, acetaldehyde concentrations were found to be increased significantly compared with 20°C and 30°C controls and the values obtained by enzymatic analysis in two wines (Table 2.4). Thus, all future derivatizations were conducted at 30°C for 30 hours.

Table 2.4 Effect of derivatization temperature on acetaldehyde concentrations in several wines. The derivatization time was 30 hours and results from the LC analysis were compared with an enzymatic test method.

Temperature (°C)	Acetaldehyde (mg l ⁻¹)			
	Riesling	Pinot Grigio	Mead Wine	Marechal Foch
20	14.9±0.4 ^a	62.4±0.8 ^a	24.5±1.0 ^a	65.4±1.0 ^a
30	15.1±0.9 ^a	62.3±0.4 ^a	26.6±0.6 ^a	65.1±0.1 ^a
50	18.1±1.1 ^a	66.0±0.7 ^a	35.8±0.8 ^b	72.0±0.7 ^b
Enzymatic	16.1±2.2 ^a	60.8±4.7 ^a	23.8±0.8 ^a	63.4±2.0 ^a
Control				

Different letters within columns indicate statistically significant differences at <0.01 confidence level.

In order to mimic the significant compositional changes taking place during transformation of musts into wine, the potential effect of glucose concentrations (12.5 to 100 g l⁻¹) on the derivatization efficiency of alpha-ketoglutarate, pyruvate and acetaldehyde at various concentrations (12.5 to 100 mg l⁻¹) was evaluated. Glucose concentrations of 50 g l⁻¹ and higher led to interferences, which reduced pyruvate and alpha-ketoglutarate recoveries by 25% and 8.5% respectively (at 100 g l⁻¹ glucose), but did not affect acetaldehyde in a statistically significant manner (Figure 2.3).

In contrast, large acetaldehyde concentrations (25 – 100 mg l⁻¹) were not found to affect the derivatization efficiencies of alpha-ketoglutarate, pyruvate and glucose over the concentration range (25 to 100 mg l⁻¹) tested.

The potential for SO₂ and red wine pigments to interfere with DNPH derivatization was also investigated. The presence of SO₂ was not found to significantly decrease derivatization of SO₂ binding compounds to DNPH within the concentration range tested (100 – 350 mg l⁻¹ SO₂, Table 2.5). Likewise, red wines treated with and without polyvinylpolypyrrolidone (PVPP) did not show statistically significant compositional differences in their SO₂ binder concentrations (Table 2.6).

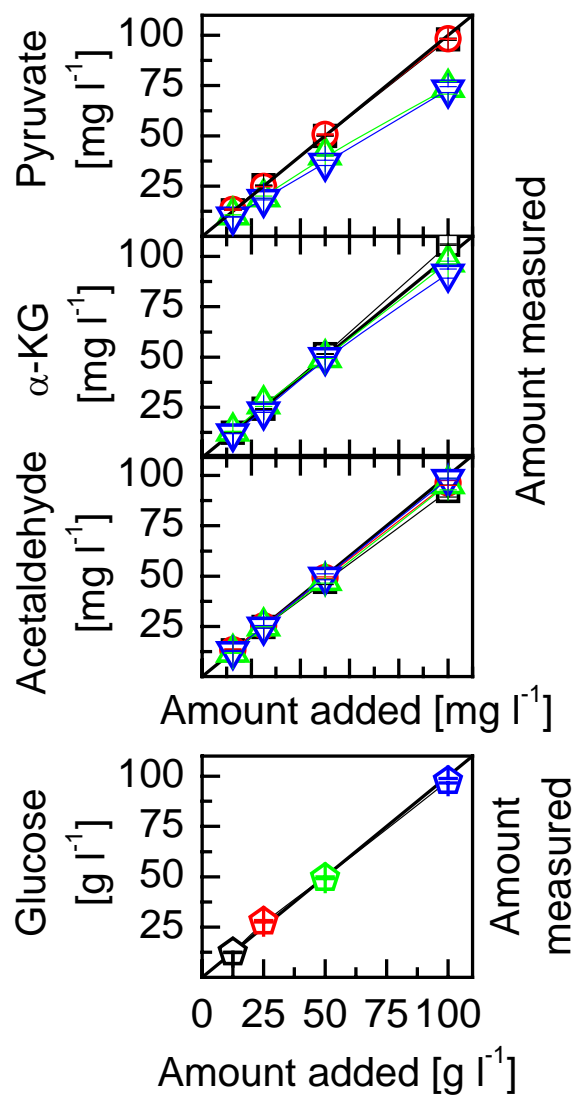


Figure 2.3 Effect of various glucose and SO₂ binder concentrations on analyte recoveries. Samples were adjusted to (□) 12.5 g l⁻¹ (○) 25 g l⁻¹ (△) 50 g l⁻¹ and (▽) 100 g l⁻¹ of glucose and the analyte concentrations indicated in the graphs.

Table 2.5 Effect SO₂ on analyte recovery. All treatments were compared to a control without SO₂ addition. Mean values (\pm SE) of triplicate determinations shown.

SO ₂ Binding Compound (mg l ⁻¹)	Recovery at total SO ₂ concentration [%]		
	100 mg l ⁻¹	200 mg l ⁻¹	350 mg l ⁻¹
Glucose (6000)	97.84 \pm 0.62 ^a	96.37 \pm 0.35 ^a	99.98 \pm 3.21 ^a
Galacturonic acid (400)	102.29 \pm 0.51 ^a	102.49 \pm 0.23 ^a	103.41 \pm 1.33 ^a
Alpha- ketoglutarate (40)	101.97 \pm 1.05 ^a	103.85 \pm 0.86 ^a	102.35 \pm 1.27 ^a
Pyruvate (40)	102.13 \pm 0.65 ^a	107.45 \pm 5.70 ^a	101.93 \pm 1.50 ^a
Acetoin (40)	103.83 \pm 1.89 ^a	106.37 \pm 1.46 ^a	104.24 \pm 3.43 ^a
Acetaldehyde (55)	103.52 \pm 2.29 ^a	104.47 \pm 2.00 ^a	101.91 \pm 1.80 ^a

Different letters within rows indicate statistically significant differences at <0.01 confidence level.

Table 2.6 Percent recovery of SO₂ binding compounds in a red wine (2006 Merlot, Finger Lakes, NY) comparing the use of PVPP to remove anthocyanins prior to spiking wine with select SO₂ binding compounds.

SO ₂ Binding Compound	Percent Recovery		P-value
	No PVPP	PVPP	
Glucose	101.7±0.7	103.1±0.1	0.145
Galacturonic acid	107.8±3.5	100.9±0.1	0.149
Alpha-ketoglutarate	96.0±0.8	95.9±1.2	0.182
Pyruvate	95.1±0.1	97.9±2.5	0.582
Acetoin	94.0±2.5	98.9±0.8	0.140
Acetaldehyde	94.9±0.1	100.7±3.9	0.069

In order to test method robustness, transferability to different stationary phases and possible co-elutions, ten commercial wines were analyzed using two different C18 phases. The linear regression and correlation analysis ($r^2 > 0.99$) demonstrated the transferability of the presented method and further verified the accuracy of quantified SO₂ binding compounds (Figure 2.4).

Further, SO₂ binders were spiked into commercial red and white wines to evaluate analyte recovery and method precision in actual sample matrices (Table 2.6). Mean percent recoveries for all compounds ranged from 92 – 104% and the precision was between 0.3% and 1.5% CV. Limits of detection for glucose, galacturonic acid, alpha-ketoglutarate and pyruvate were generally higher in red wines whereas the LOD for acetaldehyde was higher in white wines (Table 2.7).

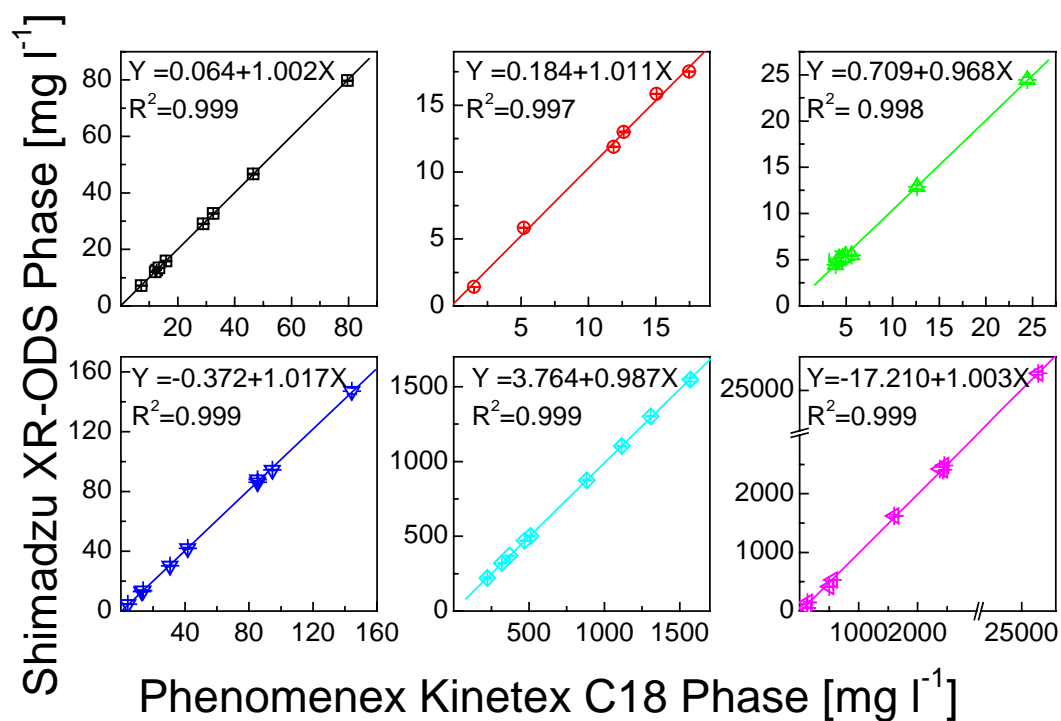


Figure 2.4 Comparison of the analytical method using two stationary phases (100mm x 3.0mm Shimadzu XR-ODS and 100mm x 4.6mm Phenomenex Kinetex C18) for the separation and quantification of (□) acetaldehyde, (○) acetoin, (△) pyruvate, (▽) α-ketoglutarate, (◇) galacturonic acid and (◊) glucose in ten commercial wines.

Table 2.7 Key chromatographic parameters for SO₂ binding compounds spiked into commercial wines (five red and white). Data shows average (\pm SE) of ten samples.

Compound	Retention Time (min)	Calibration Range (mg l ⁻¹)	Stock Standard (g l ⁻¹)	Spike (mg l ⁻¹)	Mean Sample Recovery	Recovery Range	Resolution (R _s)	LOD White wine (mg l ⁻¹)	LOD Red wine (mg l ⁻¹)	%CV
Glucose	5.587	200-25,000	125.0	6250	102.4 \pm 0.7%	102 – 103%	1.530 \pm 0.173	3.2 \pm 1.3	87.1 \pm 29.9	1.5 \pm 0.3
Galacturonate	7.516	30-2,000	20.0	500	104.4 \pm 3.5%	98 – 108%	1.717 \pm 0.400	9.3 \pm 3.8	14.7 \pm 9.8	0.5 \pm 0.2
Alpha-ketoglutarate	8.595	3-100	1.0	100	98.1 \pm 5.8%	91 – 102%	2.162 \pm 0.317	0.4 \pm 0.1	4.0 \pm 0.9	0.9 \pm 0.2
Pyruvate	9.530	3-100	1.0	100	93.3 \pm 1.6%	88 – 99%	2.453 \pm 0.129	0.2 \pm 0.1	0.1 \pm 0.1	0.4 \pm 0.1
Acetoin	10.415	2-50	0.50	50	96.5 \pm 2.5%	94 – 99%	5.074 \pm 0.900	0.1 \pm 0.1	0.1 \pm 0.1	0.6 \pm 0.2
Acetaldehyde	11.858	3-100	1.0	68	93.2 \pm 4.1%	88 – 99%	2.476 \pm 0.382	0.5 \pm 0.2	0.1 \pm 0.1	0.3 \pm 0.1

The method was applied to the determination of SO₂ binding compounds in ten wines produced in New York State. Figure 2.5 displays exemplary chromatograms from the analysis of three wines. Good peak separation and low baseline noise for target SO₂ binders were achieved. Overall, clear differences in SO₂ binder concentrations between red and white wines were found (Table 2.8). Among the wines tested, galacturonic acid and alpha-ketoglutarate were found at higher concentrations in red wines, whereas glucose, pyruvate and acetaldehyde levels were typically higher in white wines.

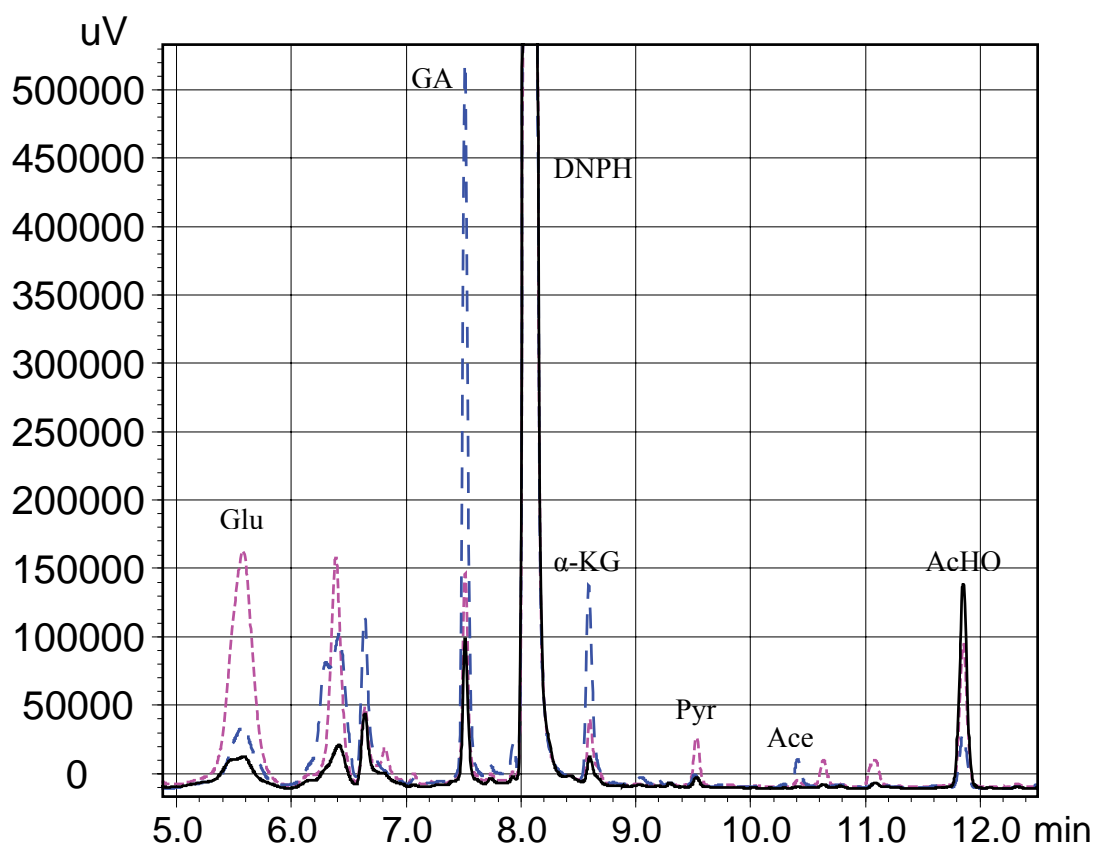


Figure 2.5 Chromatographic comparisons of DNPH derivatized SO₂ binding compounds found in 2006 Sauvignon Blanc (—), 2006 White Vinifera blend (.....) and 1998 Cabernet Franc (---). SO₂ binding compounds are eluted in the following order, (Glu) glucose; (GA) galacturonic acid; (α-KG) alpha-ketoglutarate; (Pyr) pyruvate; (Ace) acetoin; (AcHO) acetaldehyde. DNPH peak represents unreacted derivatizing reagent.

Table 2.8 Comparison of mean SO₂ binding compound concentrations in a survey of ten wines (five red, five white) from NYS.

Wine Type	Glucose (g l ⁻¹)	Galacturonic acid (mg l ⁻¹)	Alpha-ketoglutarate (mg l ⁻¹)	Pyruvate (mg l ⁻¹)	Acetoin (mg l ⁻¹)	Acetaldehyde (mg l ⁻¹)
Red	0.19±0.13	1170±104	84±20	12±1	10±2	20±5
White	4.76±1.46	270±31	15±2	19±2	8±3	41±15
P-value	0.0141	<0.0001	0.0091	0.0379	0.4384	0.2429

2.5. Discussion

This work presents and validates an improved method for the simultaneous quantification of the SO₂ binding compounds, glucose, galacturonic acid, alpha-ketoglutarate, pyruvate, acetoin and acetaldehyde using an ultra-high performance LC system.

The transformation of grape must to wine by alcoholic fermentation leads to significant matrix changes. An aqueous solution typically containing hexoses in excess of 200 g l⁻¹ is converted into a hydroalcoholic solution that may contain <1 g l⁻¹ of sugar and >15% vol. of ethanol. For the quantification of SO₂ binders, these changes pose multiple challenges. Glucose, which approximately represents half of the hexoses present in musts, binds to SO₂ with low affinity (Ribéreau-Gayon et al., 1998a). The ethanol present in fermented wines, while not binding to SO₂, will have a large effect on the concentration of acetaldehyde, which typically is the most important SO₂ binder because of its concentration and the low dissociation constant of its sulfonate. Specifically, under aerobic conditions, ethanol is oxidized to acetaldehyde in the presence of divalent metals and phenolics in wine (Danilewicz, 2003). In this work, derivatization efficiency in the presence of high glucose concentrations was carefully considered as well as sample preparation techniques limiting the *de novo* formation of acetaldehyde from ethanol oxidation.

Several previously published methods using DNPH derivatization do not disrupt the hydroxysulfonate bond, and thus quantify only the free or unbound forms of SO₂ binding compounds (Matsuura et al., 1990; Schmidt et al., 1983). In this work, the quantification of total SO₂ binder pools was achieved by adjusting samples to pH >10

to destabilize the hydroxysulfonate bond between SO₂ binders and SO₂. However, this pH increase has been reported to exacerbate oxidative reactions and *de novo* formation of acetaldehyde during sample pre-treatment (Elias et al., 2008). Elias et al. (2008) described a method that uses nitrogen gas to flush sample headspace and degas the sodium hydroxide solution for the hydrolysis of hydroxysulfonates. However, the gas flow must be carefully controlled to prevent stripping volatile SO₂ binders while concurrently preventing sample oxidation. In this work, the addition of a metal chelator during pre-treatment served as a simple alternative to inert gas blanketing for the control of oxidation reactions. EDTA was found to efficiently prevent acetaldehyde formation during sample treatment at 30°C, and can be easily and simultaneously applied to a large number of samples.

Results clearly show that derivatization kinetics between SO₂ binders and DNPH were analyte specific and reaction equilibria were reached after 30 hours under the conditions of this study. Derivatization reactions in other protocols ranged from 0.5-12 hours (Elias et al., 2008; Lea et al., 2000; Matsuura et al., 1990; Schmidt et al., 1983). Such incubation durations would be inadequate for the reproducible quantification of slow derivatizing SO₂ binders, such as glucose and galacturonic acid, which may significantly contribute to SO₂ binding in some wines. Hufnagel & Hofmann (2008) reported galacturonic acid levels of up to 1.76 g l⁻¹ in wines, and glucose concentrations in excess of 100 g l⁻¹ are common in sweet or dessert wines.

Examining the effect of glucose on analyte derivatization efficiency is crucial if the method is to be considered for analyses of musts and wines. Interestingly, in spite of a large stoichiometric excess of glucose with regards to DNPH, the recovery of glucose,

itself, was not reduced under higher glucose conditions, yet other SO₂ binders were affected. Hence, large sugar concentrations may interfere with non-glucose SO₂ binder recovery at shorter derivatization times as used in other works, too, and should be considered accordingly. With the present method, accurate analysis of all SO₂ binders could be achieved by diluting samples with glucose residues in excess of 25 g l⁻¹.

SO₂, itself, was not found to interfere with studied SO₂ binding compounds within practical and legal concentration ranges. This finding agrees with previous work done by Lea et al. (2000) who determined that reaction rates between sulfite and SO₂ binders were slow at levels up to 250 mg l⁻¹ total SO₂, and should not impact derivatization efficiency with DNPH.

In spite of the large number of analytes, UHPLC separation of SO₂ binders using the current method required only 13.5 minutes, compared to other protocols that ranged from 20-55 minutes (de Azevedo et al., 2009; Elias et al., 2008; Lea et al., 2000; Matsuura et al., 1990). The rapid analysis, allowing for higher sample throughput, was achieved by using small diameter solid core particles (2.6 µm) at high pressures (~12 MPa) that facilitate fast analyte separation while maintaining chromatographic resolution suitable for quantification. The overall analysis time of the current method including derivatization is significant, and was chosen for the comprehensive determination of SO₂ binders at practical concentration ranges, while achieving high method precision. For projects where comprehensive analysis of the SO₂ binder pool by a single method is advantageous, the current protocol allows for an efficient utilization of analytical equipment and manual labor since the high stability of DNPH

adducts allows for simultaneous derivatization of numerous samples without adherence to strict analysis times.

Where provided for similar methods, analytical precision was between 0.5% and 10.6%CV (de Azevedo et al., 2009; Elias et al., 2008; Matsuura et al., 1990). The precision for the current method was from 0.3 to 1.5 %CV. The chromatographic resolution values determined for two UHPLC columns revealed that both were acceptable for quantification ($R_s > 1.5$) (Harris, 2003) though, the solid core particle column yielded higher R_s values for all SO₂ binders. Comparison of chromatographic resolution with similar methods was not possible due to lack of available data. The calibration ranges for SO₂ binders chosen for this method were based on levels commonly found in red and white wines, thus eliminating the need to dilute most wine samples prior to analysis, a common requirement in other protocols (de Azevedo et al., 2009; Elias et al., 2008).

Quantification of SO₂ binders in ten commercial wines revealed that red wines typically had high levels of galacturonic acid and alpha-ketoglutarate, which likely results from increased grape skin contact during vinifications (Ribéreau-Gayon et al., 1998b; Watanabe et al., 1979). Conversely, pyruvate and acetaldehyde were lower in red wines, most likely because of their degradation by lactic acid bacteria during malolactic fermentation (Flamini et al., 2002; Osborne et al., 2000; Zaunmuller et al., 2006).

2.6. Conclusions

An improved method for the separation and quantification of significant SO₂ binding compounds after derivatization with DNPH was presented. Compared with similar

methods the analytical protocol greatly simplified sample pre-treatment through the use of EDTA to prevent *de novo* formation of SO₂ binding compounds, especially acetaldehyde. Incubation time and temperature were optimized to provide the comprehensive analysis of significant SO₂ binding wine carbonyls, including glucose and galacturonic acid. SO₂ residues and red wine pigments did not interfere with analyte recoveries, while high glucose concentrations reduced the derivatization efficiency of some SO₂ binding compounds. UHPLC separation of DNPH derivatives using a solid core C18 phase yielded chromatographic resolution values suitable for quantification while reducing analysis time. Method transferability to a different reverse phase was demonstrated. SO₂ binding compounds could be quantified over a practical dynamic range while maintaining high precision and low limits of detection. The improved method is valuable for the comprehensive analysis of total SO₂ binder pools in enological samples.

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CHAPTER 3: RESULTS FROM THE STUDY OF SO₂ BINDING CARBONYLS IN 237 COOL CLIMATE TABLE WINES FROM NEW YORK STATE, AND THEIR APPLICATION FOR THE REDUCTION OF PRESERVATIVE SO₂ LEVELS

3.1. Abstract

Sulfur dioxide (SO₂) is an important preservative commonly used in winemaking. High concentrations of SO₂ binding wine carbonyls limit sulfite efficacy resulting in higher total SO₂ additions, which may lead to final wine SO₂ concentrations that exceed limits permitted by law and pose health risks for sensitive consumers. Six SO₂ binding compounds (acetaldehyde, pyruvate, α -ketoglutaric acid, galacturonic acid, glucose and acetoin) were quantified in 237 cool climates wines by HPLC with pre-column derivatization to DNPH. The most important SO₂ binders (mean \pm SE) in red and white wines were acetaldehyde (red, 25 \pm 3 mg l⁻¹; white, 40 \pm 3 mg l⁻¹), pyruvic acid (red, 14 \pm 2 mg l⁻¹; white, 25 \pm 2 mg l⁻¹), α -ketoglutaric (red, 74 \pm 4 mg l⁻¹; white, 31 \pm 3 mg l⁻¹) and galacturonic acids (red, 810 \pm 51 mg l⁻¹; white, 267 \pm 13 mg l⁻¹). Overall, acetaldehyde was identified as the most important SO₂ binder because of its binding power and mean concentrations. Acetaldehyde formation from the involuntary oxidation of ethanol during the post-fermentation stages likely is responsible for the large differences in acetaldehyde concentrations in products from individual wineries and represents the most efficient target for efforts directed at reducing SO₂ binders. Post-fermentation wine handling and bottling were identified as critical control points for the formation of acetaldehyde.

3.2. Introduction

Sulfur dioxide (SO₂) is an effective and low cost additive for the preservation of wines and other food products (Doyle and Beuchat, 2007). It is an anti-microbial (Hood, 1983), a potent anti-oxidant (Danilewicz, 2003) and has anti-enzymatic activities (Main and Morris, 1991; Wedzicha et al., 1991). Unfortunately, it poses health risks for sensitive consumers. The US FDA estimates that as many as 1% of the U.S. population show an increased degree of sensitivity to sulfites (Papazian, 1996). In particular, asthmatics appear to be especially sensitive, with estimates that as many as 5% may risk adverse reactions upon ingestion of sulfites (Snellen and Schaafsma, 1992; Vally et al., 1999). Minimally processed foods and wines have been increasingly popular with consumers in recent years (Azabagaoglu et al., 2007). In the U.S., sales of certified organic foods have increased from \$78 million in 1980 to approximately \$6 billion in 2000, with an average annual increase of 24% during the 1990s (Hughner et al., 2007). The significant increase in the number of organic wineries in Europe (Azabagaoglu et al., 2007) and the steady increase in “sustainable” or “fresh” (kegged wines without added SO₂) wines with lower SO₂ concentrations in the United States and Canada may also be indicative of the increasing consumer interest in minimally processed wines.

Total preservative SO₂ concentrations in wine are dependant on carbonyl and keto compounds, which are known to form covalent adducts (sulfonates) with SO₂. Bound forms of SO₂ are thought to have decreased preservative activity compared to free SO₂ (Rankine, 1968b). Hence, wines with high concentrations of SO₂ binding compounds typically require increased SO₂ additions for equivalent preservative action.

Acetaldehyde is an important SO₂ binding compound found in wine, primarily due to the low dissociation constant of its sulfonate ($K_d = 1.5 \times 10^{-6}$) (Ribéreau-Gayon et al., 1998a). Other SO₂ binding compounds including pyruvate and α -ketoglutaric acid may also have a significant effect on bound SO₂ levels (Ribéreau-Gayon et al., 1998a). Despite the importance of key SO₂ binding compounds for wine preservation, few studies have assessed their concentrations across a large sampling of commercial table wines. The aim of this work was to quantify common levels of major SO₂ binding compounds for the purpose of SO₂ reduction in commercial table wines to ensure adherence to regulatory limits, and to meet consumer demands for minimally preserved wines. The SO₂ binding compounds acetaldehyde, pyruvic, α -ketoglutaric, and galacturonic acids, glucose and acetoin were quantified in 237 cool climate wines from New York State by HPLC with pre-column derivatization to DNPH. A two-year survey of industrial practices in New York State wineries identified critical control points for the formation of acetaldehyde and evaluated opportunities for the reduction of SO₂.

3.3. Materials and Methods

3.3.1. Collection of commercial wines and sampling

For the survey of wine carbonyls, 237 wines were collected from commercial wineries throughout New York State. Table 3.1 provides a classification of these wines by wine color and producing region. Vintage wines ranged from 1998 to 2006 with 61% obtained from the 2005 and 2006 vintages. All wines were sampled anaerobically and samples were kept frozen until analysis.

Table 3.1 Overview of wine sample origin and color

Region	Red	White	Total
LE	1	7	8
FL	63	84	147
HV	9	9	18
LI	28	24	52
Other	9	3	12
Total	110	127	237

(LE) Lake Erie, (FL) Finger Lakes, (HV) Hudson Valley, (LI) Long Island

3.3.2. Time course of acetaldehyde during vinifications

For the analysis of acetaldehyde concentrations during commercial vinifications, must/wine samples were taken at different winemaking stages at eight participating wineries across New York State. Every winery supplied samples of two vinifications over two vintages and all samples were kept and shipped frozen until analysis.

3.3.3. Chemicals and preparation of reagents for SO₂ binder analysis

All chemicals were of analytical grade and obtained from Thermo Fisher Scientific (Pittsburgh, PA). ASTM Class I water was prepared using a water purification system (Arium 611UV, Sartorius, Germany). The concentration of non-carbonyl analytes reported in this work were determined by flow injection analysis with spectrophotometric detection (FOSS WineScan; Foss North America, Eden Prairie, MN, USA). DNPH had a purity of 97% (as dry weight) and contained 30-40% water to reduce the explosion hazard. The derivatizing reagent was prepared by dissolving DNPH in acetonitrile adjusted to pH 1.5 with perchloric acid to obtain an 11 mM DNPH solution. For sample pre-treatments, an aqueous solution of 86 mM EDTA in 1 M sodium hydroxide was prepared and degassed (Aquasonic Model 150D, VWR International, PA) for 20 minutes. Solutions were stored up to one week at 4°C.

3.3.4. Derivatization procedure

Derivatizations were conducted in 2.0 ml glass HPLC vials with Teflon caps (National Scientific, TN). For sulfonate hydrolysis, 200 µl of sodium hydroxide/EDTA solution were added to the vial, followed by 100 µl of sample wine and mixing. After 10 minutes, 200 µl of 1M perchloric acid were added followed by 800 µl of the derivatizing reagent. After mixing, the solution was allowed to react for exactly 30 hours at 30.0±0.1°C and then promptly cooled to 4°C until analysis. Derivatized samples thus prepared were stable for up to five days.

3.3.5. HPLC Analysis

An ultra high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AD XR pumping unit, a DGU-20A₃ degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven, and a SPD-20A UV/VIS detector were used for separation and analysis of DNPH derivatized wine carbonyls. Data acquisition and analysis was performed with the LCSolution software (1.23). Solvent A consisted of water acidified to pH 2.50 ± 0.01 using perchloric acid. Solvent B was HPLC grade acetonitrile. All solvents were filtered prior to utilization (0.22 μm , nylon, Millipore, Ireland). Samples were held at 4°C in the autosampler and 5.0 μl of sample injected directly. Separation occurred on a Phenomenex Kinetex C18 stationary phase (100 x 3.0 mm) with 2.6 μm particle size held at 37°C with a flow rate of 0.75 ml min⁻¹. The analytes were quantified at 365 nm using external calibration standards with linear regression analysis. The separation gradient is shown in Table 3.2.

Table 3.2 Gradient program for the chromatographic separation of DNPH derivatized SO₂ binding compounds using a Phenomenex Kinetex C18 column (100 x 3.0 mm).

Time (min)	Solvent A	Solvent B
0.00	85	15
4.25	82	18
4.75	75	25
6.50	62	38
12.00	40	60
12.50	10	90
13.25	85	15

3.3.6. Statistical analysis

Student's t-tests were conducted with JMP 7.0 (SAS, North Carolina) to determine statistical significance between sample populations at the 0.05 confidence level.

3.4. Results

Table 3.3 shows the average concentrations of SO₂ binding compounds in NYS table wines and their ranges according to wine color. Except for acetoin, statistically significant differences were found between the mean concentrations of all other SO₂ binders in red and white table wines. Glucose, pyruvic acid and acetaldehyde concentrations were found to be 240%, 79% and 60% higher in white wines compared with red, respectively. Conversely, the concentrations of galacturonic and α -ketoglutaric acids were 203% and 139% higher in red wines, respectively (Table 3.3). Figure 3.1 illustrates the concentration distributions of the six selected SO₂ binding compounds, contrasted by wine color. In red wines, galacturonic acid and α -ketoglutaric acid concentrations were distributed across a larger concentration range than in whites where the data was more clustered. Alternatively, pyruvic acid, glucose and acetaldehyde concentration data was distributed over larger ranges in white wines compared to reds.

The mean concentrations of SO₂ binders in red and white table wines (Table 3.3) were used to estimate the total bound SO₂ levels in stabilized wines and the percentage of the respective sulfonate species (Table 3.4). For this calculation, the dissociation constants reported by Ribéreau-Gayon et al (1998b) and Blouin (1966) were used, and the wine was assumed to have 30 mg l⁻¹ of free SO₂ and no other SO₂ binders. Under these assumptions, white table wines were calculated to have 15 mg l⁻¹ more bound

SO₂ than reds. Acetaldehyde was calculated to account for over two-thirds of the bound SO₂ in white table wines, with minor contributions by pyruvic acid and α -ketoglutaric acid. While acetaldehyde was also found to be the major contributor to bound SO₂ in red table wines, the percentage of other binders was more prominent. Also, α -ketoglutaric acid was found to be of higher importance than pyruvic acid in red wines, while the contrary was found for whites.

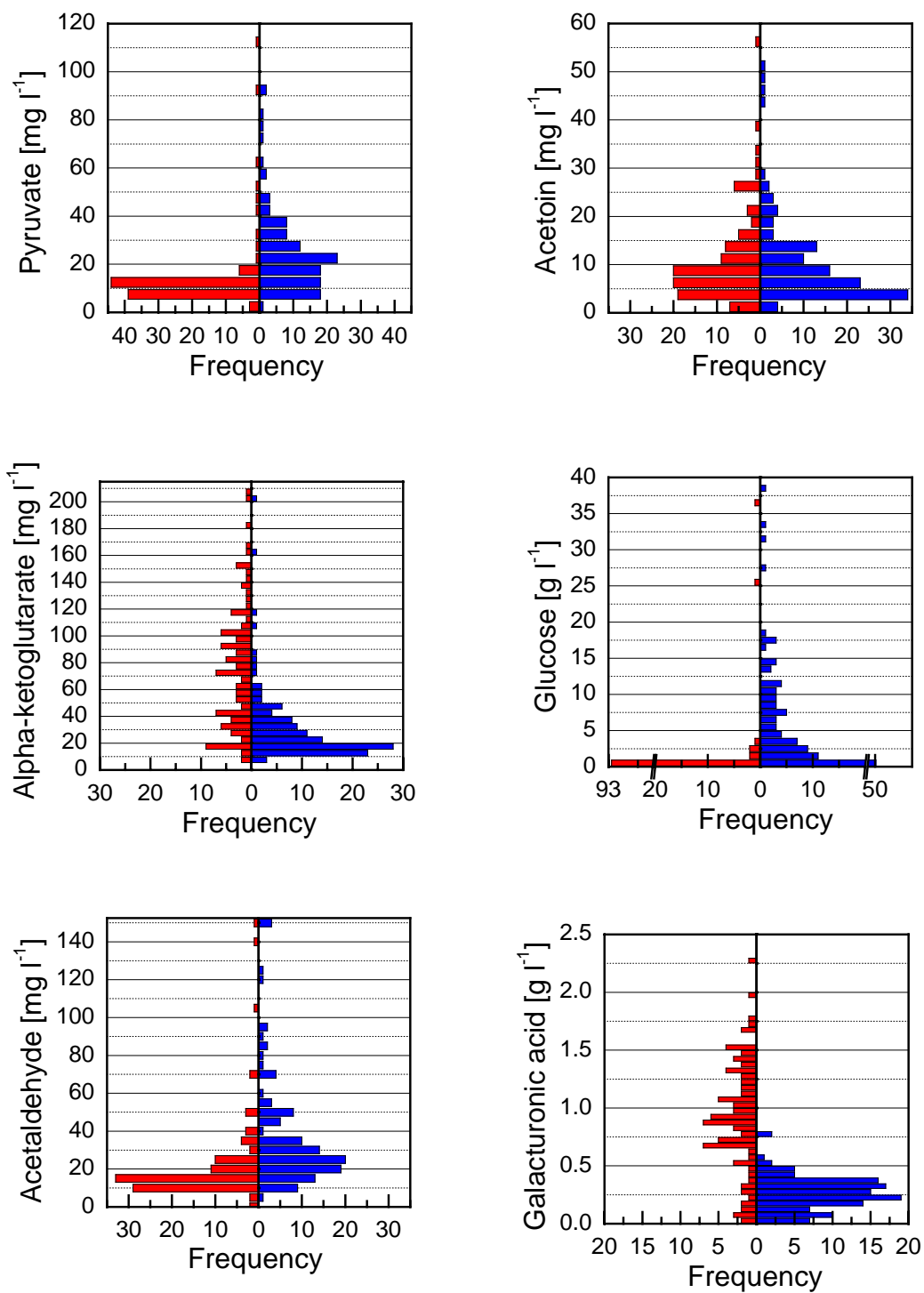


Figure 3.1 Frequency graphs for SO₂ binders in 237 NYS wines contrasting red (red bars) and white (blue bars) wines.

Table 3.3 Comparison of mean values (\pm SE) of SO₂ binders in commercial table wines from NYS. P-values indicate statistical differences between red and white wines for each compound.

SO ₂ Binding Compound	Red Wine (mg l ⁻¹)			White Wine (mg l ⁻¹)			P-value
	Mean	Median	Range	Mean	Median	Range	
Glucose	1,400 \pm 770	nd	nd - 69,110	4,750 \pm 648	1637	nd - 38,362	0.0033
Galacturonic Acid	810 \pm 51	854	7 - 2,274	267 \pm 13	263	19 - 768	<0.0001
α -ketoglutaric acid	74 \pm 4	71	7 - 208	31 \pm 3	22	6 - 202	<0.0001
Pyruvic acid	14 \pm 2	11	nd - 113	25 \pm 2	22	5 - 92	<0.0001
Acetoin	11 \pm 1	8	1 - 57	10 \pm 1	7	1 - 50	0.7897
Acetaldehyde	25 \pm 3	17	nd - 211	40 \pm 3	29	7 - 240	0.0002

N = 110 and 127 for red and white wines respectively, ND = Not detected.

Table 3.4 Prediction of the contribution of wine carbonyls to bound SO₂ in red and white wines. The relative share of the bound SO₂ was based on mean carbonyl concentrations measured in 110 red and 127 white wines, the dissociation constants of the respective sulfonates, and assuming 30 mg l⁻¹ of free SO₂. Mean values ±SE shown.

SO ₂ binder	Percent of wine carbonyl bound to SO ₂ [%]	
	Red	White
Acetaldehyde	55.4±4.3	71.5±4.3
Pyruvic acid	11.6±1.6	16.7±1.6
α-ketoglutaric acid	22.6±1.2	7.6±0.9
Galacturonic acid	10.4±0.6	2.7±0.2
Glucose	nil	1.5±0.3
Total	100	100
Bound SO ₂	65.8 mg l ⁻¹	81.2 mg l ⁻¹

Figure 3.2 displays winery specific mean acetaldehyde concentrations. Overall, mean acetaldehyde values were found to vary greatly between wineries. 73% and 59% of the wineries had mean acetaldehyde concentrations below 60 mg l⁻¹, and 40 mg l⁻¹, respectively. There was a trend towards larger acetaldehyde concentration variations within the samples provided by wineries with mean acetaldehyde concentrations exceeding 60 mg l⁻¹.

To study acetaldehyde kinetics during commercial vinifications, white table wine samples from 32 vinifications provided by eight wineries across New York State were analyzed. Figure 3.3 shows the acetaldehyde concentrations measured after individual winemaking steps, and their relative change between these steps. Values were averaged across wineries.

Contact with yeast lees and malolactic fermentation lead to considerable acetaldehyde reductions across all vinifications. Conversely, most post-fermentative winemaking steps resulted in moderate to important acetaldehyde increases. In 2010, the post-fermentative acetaldehyde kinetics did not exhibit the large increase observed during the 2009 vintage, where mean acetaldehyde concentrations increased by over 20 mg l⁻¹ from the conclusion of MLF to the bottled wine.

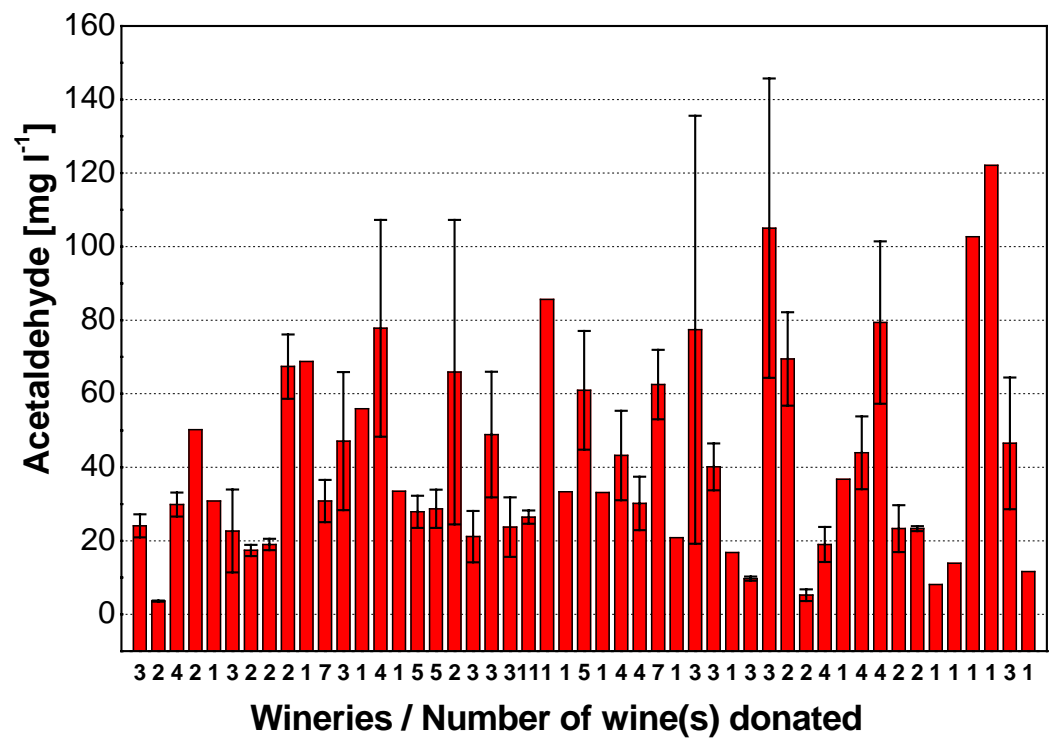


Figure 3.2 Mean concentration of acetaldehyde (\pm SE) from each surveyed winery. Frequency of wines analyzed per winery is shown on the x-axis.

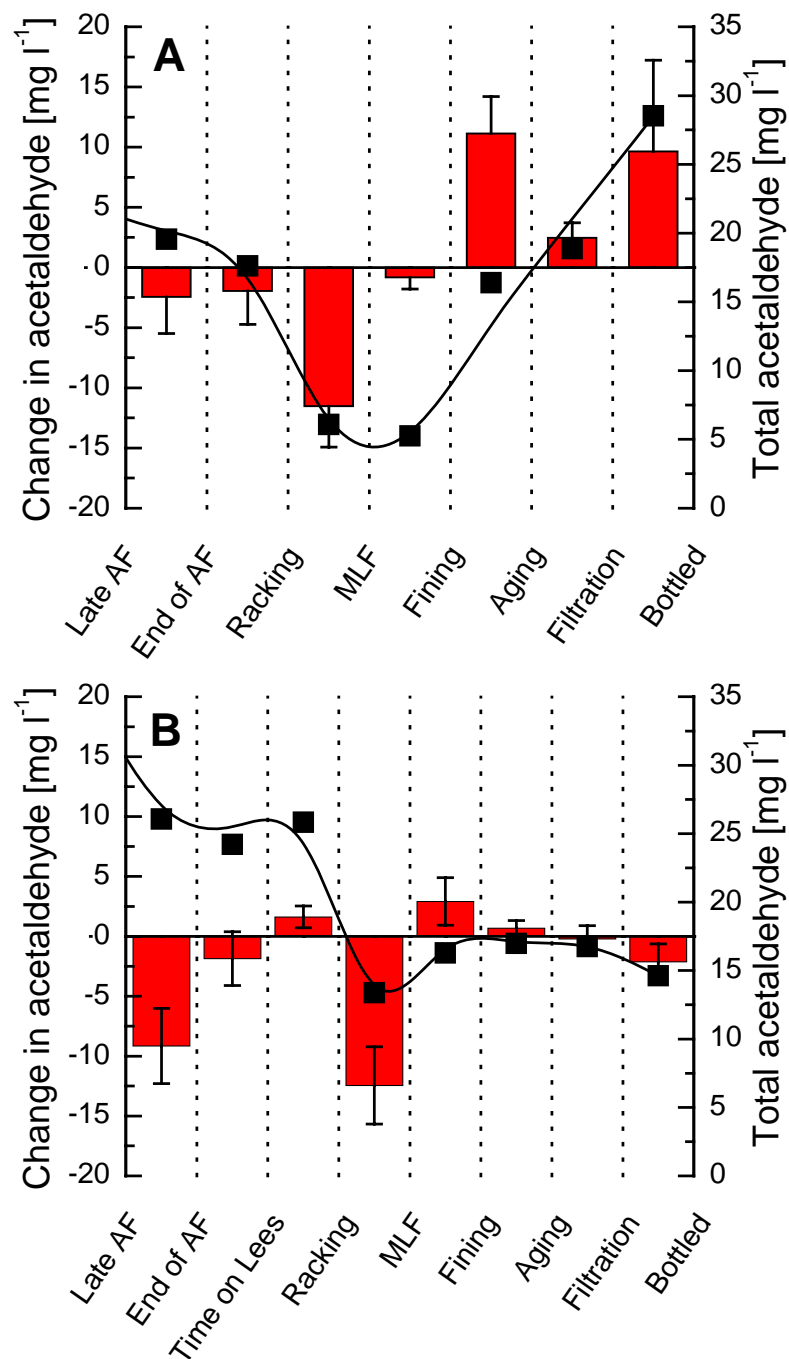


Figure 3.3 Course of acetaldehyde concentrations during vinifications in eight NYS wineries and relative acetaldehyde changes between winemaking stages in (A) 2009 and (B) 2010. Averages from eight wineries \pm SE displayed. Values were obtained from samples taken after the indicated winemaking step.

3.5. Discussion

SO₂ has been used for centuries during vinifications, and is important for the preservation of wine and other food products (Doyle and Beuchat, 2007). Increased regulatory pressure and consumer perception (Magnusson et al., 2001; Smith, 2002) are prompting the re-evaluation of SO₂ management strategies. An efficient approach to limit SO₂ usage during winemaking is by reducing wine carbonyls, which bind to SO₂ and reduce free and active SO₂ levels (Rankine, 1968b; Ribéreau-Gayon et al., 1998a).

A survey of 237 table wines from across New York State revealed important differences between SO₂ binder concentrations in red and white wines, which reflect the fundamental differences in the vinification protocols. Most red wines are produced by fermentation in the presence of grape skins to induce release of color and aroma compounds (Ough, 1992). This explains the larger concentrations of galacturonic acid, a monomer of grape skin pectins, extracted during red wine fermentations. Sponholz and Dittrich (1985) reported concentration ranges for galacturonic acid of 116 to 1048 mg l⁻¹ in cool climate whites, which are similar to those presented here. However, the maximum galacturonic acid values in red wines reported in the current study were over 1 g l⁻¹ higher than those published by Sponholz and Dittrich (1985) (381-1200 mg l⁻¹). The high values reported here may be attributable to the presence of wines produced from native American grapes, which are known to have significantly higher pectin levels than European *Vitis vinifera* grapes (Rice, 1974). The large variation of galacturonic acid values among the samples observed indicate

differences in grape variety and quality, extraction duration and efficiency, and the addition of pectolytic enzymes (Lao et al., 1996).

A limited number of studies are available with regards to α -ketoglutaric acid levels in table wines. In sweet dessert wines, concentration ranges of 70-273 mg l⁻¹ (Ribéreau-Gayon et al., 1998a) and 78-248 mg l⁻¹ (Barbe et al., 2000) have been reported. Rankine (1968a) reported an average of 53 mg l⁻¹ with a range of 6-135 mg l⁻¹ in Australian whites. Overall, α -ketoglutaric acid concentrations measured in this work corresponded with these studies. However, red wines were found to have average α -ketoglutaric acid levels that were over twice as high than those found in whites ($p < 0.0001$). Watanabe et al. (1979) reported higher α -ketoglutaric acid concentrations in skin fermented red wines, but the reasons for the increased values were not elucidated.

In contrast to whites, most red wines undergo malolactic fermentation (MLF) that typically occurs after alcoholic fermentation, resulting in the decarboxylation of L-malic to L-lactic acid and wine deacidification. MLF is carried out by wine lactic acid bacteria, which are known to degrade some carbonyls including pyruvic acid and acetaldehyde (Flamini et al., 2002; Osborne et al., 2000; Zaunmuller et al., 2006) in addition to malic acid. In this study, the highest pyruvic acid and acetaldehyde concentrations were measured in wines with < 1 g l⁻¹ of L-lactic acid, most of which were whites. Among those wines exceeding 3.0 g l⁻¹ of L-lactic acid, the maximum levels for pyruvic acid and acetaldehyde were found to be 13 mg l⁻¹ and 50 mg l⁻¹, respectively (Supporting data). Sponholz (1982) reported acetaldehyde levels in cool climate whites and reds ranging from 7 to 67 mg l⁻¹ (mean 29.2 mg l⁻¹). More recently,

in a survey of 213 European wines for acetaldehyde, Lachenmeier & Sohnus (2008) reported mean concentrations of 34 mg l^{-1} with values ranging from 0 to 210 mg l^{-1} . Comparatively, mean acetaldehyde concentrations across all table wines in this study were almost identical at $33 \pm 3 \text{ mg l}^{-1}$ (range of 0 to 240 mg l^{-1}). However, this work demonstrated that differences existed between red and white wines that were of practical and statistical significance.

Pyruvic acid levels ranged between 0 – 113 mg l^{-1} in reds and 5 – 92 mg l^{-1} in whites in this work. The pyruvic acid concentration ranges in white wines were similar to those reported by Rankine (1968a) who observed values from 21- 147 mg l^{-1} in laboratory fermented commercial juices. However, the mean value in the study by Rankine (1968a) was over twice as high compared with the results obtained here. Yeast pyruvate formation may be influenced by the nutritional status of the musts. In sweet French wines, pyruvate concentrations ranged from traces to 51 mg l^{-1} in wines supplemented with thiamine. The same wines reached pyruvic acid levels of up to 330 mg l^{-1} without nutritional supplementation (Ribéreau-Gayon et al., 1998a). The low mean levels of pyruvate found in white wines in this study may be indicative of the more widespread application of yeast nutrients today.

In agreement with a previous study by Weeks (1969), the most important carbonyl SO_2 binders in white table wines were calculated as being acetaldehyde, followed by pyruvic and α -ketoglutaric acids. Not surprisingly, these compounds have some of the lowest dissociation constants of quantitatively important wine carbonyls. However, in red table wines α -ketoglutaric acid was found to be more relevant for SO_2 binding, and the weight of galacturonic acid was similar to pyruvic acid. Accordingly, studies

aimed at reducing SO₂ binding in reds should focus on the role of skin maceration and its effects on these compounds.

Considerable glucose residues may remain in wines, though glucose is not typically a significant binder of SO₂ due to the high dissociation constant of its sulfonate. In this study, glucose was found in large concentrations especially in white wines, which is typical for cool climate wines to achieve a balanced acidity.

The coupled co-oxidation of ethanol in the presence of atmospheric oxygen leads to chemical formation of acetaldehyde (Danilewicz, 2003; Elias and Waterhouse, 2010). White wines that have not undergone MLF and suffered from oxidation are therefore most likely to display high acetaldehyde levels, requiring more SO₂ for stabilization. Because of the risk of oxidative formation of acetaldehyde, and because acetaldehyde was found to be the most important SO₂ binder in both red and white table wines, this study focused on the production of cool climate white table wines to identify critical control points for acetaldehyde formation and reduction during commercial vinifications.

Across both vintages studied, late alcoholic phase yeast metabolism contributed to a significant decrease in acetaldehyde levels. This finding is supported by results obtained by Jackowetz et al. (2011) who observed that yeast were able to reutilize acetaldehyde rapidly in the second fermentation phase. Following alcoholic fermentation, contact with yeast lees further reduced acetaldehyde levels. A study by Madrera al. (2008) observed that yeast lees contact led to a continual decrease in acetaldehyde levels from 27 mg l⁻¹ to 21 mg l⁻¹ in cider over a 15-month period. The reduction of acetaldehyde during MLF was significant over both vintages studied, and

this finding agrees with current knowledge of acetaldehyde biotransformation during MLF (Flamini et al., 2002; Osborne et al., 2000). In the first year of the study (2009), post-fermentative vinification stages contributed significantly to *de novo* acetaldehyde formation. Aging and bottling operations represented critical control points, with some contribution from filtration.

Comparison of the two vintages identified clear differences in the course of acetaldehyde concentrations. Participating wineries were able to reduce acetaldehyde formation during post-fermentative wine handling, thus reducing mean residual acetaldehyde levels from 29 mg l⁻¹ in 2009 in bottled wines to 15 mg l⁻¹ in 2010. This improvement suggests that extension efforts and winemaker education may further reduce acetaldehyde concentrations and help achieve lower SO₂ levels.

3.6. Conclusions

Through a survey of concentrations of important SO₂ binding wine carbonyls in over 200 wines from New York, this study has provided data for benchmarking, and a starting point for further studies on the reduction of SO₂ levels in wines. The identification of the carbonyl compounds most important for SO₂ binding suggest that red skin maceration, yeast nutrition, yeast lees contact time, and malolactic fermentation are major enological factors allowing to control the overall SO₂ binding in table wines. A two-year survey of eight wineries indicates that targeted extension activities might assist in reducing SO₂ binder concentrations, especially those caused by post-fermentative oxidations, which are involuntary and do not depend on wine style.

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CHAPTER 4: MULTIFACTORIAL ANALYSIS OF ACETALDEHYDE KINETICS DURING ALCOHOLIC FERMENTATION BY *SACCHAROMYCES CEREVISIAE*

4.1. Abstract

Acetaldehyde is the terminal electron acceptor in the alcoholic fermentations (AF) of *Saccharomyces cerevisiae*. Quantitatively the most important carbonyl by-product, it has relevance for ethanol production yields as well as product stabilization and toxicology. The aim of this study was to investigate the effect of various enological parameters on acetaldehyde kinetics during alcoholic fermentations. Two commercial yeast strains were tested in two grape musts and the pH, temperature, SO₂ and nutrient addition were varied. All incubations had uniform kinetics where acetaldehyde reached an initial peak value followed by partial reutilization. Peak and residual acetaldehyde concentrations after 15 days correlated well and ranged from 62 to 119 mg l⁻¹ and 22 and 49 mg l⁻¹, respectively. Several factors had a significant effect on peak and/or final acetaldehyde levels. The results allowed for estimation of the acetaldehyde increase caused by SO₂ addition to 366 µg of acetaldehyde per mg of SO₂ added to the must. The course of the final fermentation phase was shown to determine acetaldehyde residues. A novel relationship was discovered between the time of occurrence of peak acetaldehyde concentrations and the divergence of glucose and fructose degradation rates, which may be of relevance for the overall fermentation dynamics.

4.2. Introduction

Acetaldehyde is a reactive, low molecular weight, flavor active compound found in a variety of foods and beverages such as cheese (Weerkamp et al., 1996), yogurt

(Bottazzi et al., 1973), beer (Margalith, 1981) and wine (Bottazzi et al., 1973; Dittrich and Barth, 1984; Margalith, 1981; Weerkamp et al., 1996). It is quantitatively the most important carbonyl compound produced during alcoholic fermentations (AF) with final concentrations typically varying between 10 and 200 mg l⁻¹ (McCloskey and Mahaney, 1981), and a sensory threshold from 100 to 125 mg l⁻¹ in wine for free acetaldehyde (Zoecklein et al., 1995). Acetaldehyde may also be produced post-AF through chemical oxidation of ethanol when wine is exposed to air (Danilewicz, 2003). High acetaldehyde concentrations impart aromatic attributes of green apples, fresh cut grass and walnuts (Margalith, 1981). These sensory descriptors are typical of wines such as Sherry or Vin Jaune (Cullere et al., 2007), which usually contain higher levels of acetaldehyde, but are undesired in most other wines.

In addition to its sensory qualities, acetaldehyde is a strong binder of the preservative sulfur dioxide (SO₂) (Burroughs and Sparks, 1973). SO₂ binding reduces the sensory effect of acetaldehyde, but also the functional properties of SO₂. Specifically, wines high in acetaldehyde will require more SO₂ to achieve adequate levels of free or active SO₂, since bound SO₂ does not have the same antimicrobial (Hood, 1983), antienzymatic (Main and Morris, 1991) or antioxidant properties (Danilewicz, 2003) as free SO₂. Increased levels of SO₂ have been associated with adverse reactions in sensitive consumers that may resemble asthmatic responses (Snelten and Schaafsma, 1992). Acetaldehyde, itself, has also been suspected to cause long-term adverse effects in consumers. A recent study concluded that acetaldehyde in alcoholic beverages could lead to saliva concentrations above levels previously regarded as potentially

carcinogenic (Lachenmeier and Sohnius, 2008) and may lead to increased lifetime cancer risks (Lachenmeier et al., 2009).

During AF, acetaldehyde will arise from yeast metabolism of sugars via the action of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Venturin et al., 1995; Wang et al., 2006). Generally, acetaldehyde reaches a peak value during the early fermentation phases, and is then partly reutilized by yeast (Cheraiti et al., 2010; Osborne et al., 2006; Wang et al., 2006; Weeks, 1969). Acetaldehyde production by a variety of genera and species of wine yeast has been described in several studies (Cortes et al., 1998; Herraiz et al., 1989; Longo et al., 1992; Millán and Ortega, 1988; Rankine and Pocock, 1969; Romano et al., 1994; Weeks, 1969) and reviewed by Liu and Pilone in 1998 (Liu and Pilone, 2000). However, most studies only considered final acetaldehyde levels, which may depend on the fermentation conditions and yeast lees contact time, and potential acetaldehyde production from oxygen ingress was not controlled. Finally, there is no work considering acetaldehyde production and degradation kinetics of *S. cerevisiae* starters currently available and the effect of common winemaking parameters.

The aim of this study was to study the effect of six enologically relevant factors on microbial acetaldehyde kinetics during AF. Specific factors studied were the grape variety, yeast strain, SO₂ addition, nutrient supplementation, fermentation temperature and pH level. The kinetics of yeast growth, as well as acetaldehyde, glucose and fructose concentrations were monitored throughout AF. All incubations were carried out at laboratory scale and involuntary oxidation was prevented by anaerobic

fermentation handling and monitored by non-invasive on-line measurement of dissolved oxygen.

4.3. Materials and Methods

4.3.1. Microorganisms

Saccharomyces cerevisiae var. *bayanus* strains “EC1118” and “DV10” are commercially available from Lallemand Inc. (Montréal, Canada) and were stored according to manufacturer's recommendations.

4.3.2. Grape must, general inoculation procedures and sampling

The analytical profiles of the thermovinified Sauvignon Blanc and Gewürztraminer musts (Kamil EX-IM, Canada) are shown in Table 4.1. Yeast inoculations were conducted in accordance with the supplier's recommendations using 250 mg l⁻¹ of yeast and 250 mg l⁻¹ of yeast rehydration nutrient (GoFerm, Lallemand, Canada) during rehydration. Grape must was distributed into forty sterile 250 ml glass bottles with fermentation locks and filled to 200 ml for incubations. A total of twenty different fermentations were carried out in duplicate. Samples from the fermenting musts were taken on a daily basis and immediately frozen (-18°C) for future analysis. Food grade nitrogen (Airgas, USA) was used to flush the headspace of cultivation bottles throughout the experiment.

Control fermentations were defined as the following: 30 mg l⁻¹ SO₂ addition (added as potassium metabisulfite NF/FCC, Fisher Scientific, USA), pH of 3.1, 20°C fermentation temperature and 250 mg l⁻¹ complex yeast nutrient (Fermaid K, Lallemand, Canada) addition. Non-control fermentations modified SO₂ addition (no

SO₂ added), yeast nutrients (no yeast nutrient added), pH (3.6) and temperature (12°C) parameters.

Table 4.1 Analytical profile of two white grape musts.

	Brix	pH	Titrateable Acidity (Tartaric Acid g l^{-1})	Total YAN (mg l^{-1} of Nitrogen)	Total SO_2
Sauvignon Blanc	21.3	3.1	7.1	87.9	0.0
Gewürztraminer	21.0	3.1	6.5	112.78	0.0

4.3.3. Analytical methods and statistical analysis

Throughout AF, yeast growth was monitored by measuring the optical density at 650 nm using a spectrophotometer (LKB Biochrom, UK). SO₂ was measured iodometrically by the Ripper procedure (Amerine and Ough, 1974). Glucose, fructose and total acetaldehyde (SO₂ bound and unbound) were measured enzymatically using a commercial test kit (Megazyme, Ireland). Dissolved oxygen was quantified non-invasively using a fluorescence lifetime quenching dissolved oxygen meter (Presens, Germany). Titratable acidity was determined by titration of must with 1M NaOH using phenolphthalein as an indicator. Total yeast assimilable nitrogen (YAN) was calculated as the sum of primary amino acids and total ammonia. Primary amino acid content was quantified using the NOPA method (Dukes and Butzke, 1998) and ammonia was quantified using an ion selective electrode.

Standard least squares regression and ANOVA analysis were conducted with JMP 7.0 (SAS, North Carolina) to test for significance of recorded results. All parameters and interaction factors were considered during multifactorial data analysis.

4.4. Results

Figure 4.1 illustrates the fermentation profile of two exemplary experiments. The dissolved oxygen concentrations did not increase beyond 0.2 mg l⁻¹ in any of the fermentations. Across all fermentations and regardless of the yeast strain, microbial growth rates were slower in Sauvignon Blanc fermentations compared with Gewürztraminer where stationary phase was reached after only 3.9±0.7 days compared to 5.2±1.1 days in Sauvignon Blanc. The sugar degradation kinetics displayed glucophilic behavior and residual sugar levels ≤5g l⁻¹ were obtained in all

experiments, except for fermentations at 12°C where sugar residues of 10-38 g l⁻¹ remained (Table 4.2). Acetaldehyde concentrations increased rapidly during lag and the onset of growth phases (Figure 4.1), and peak values were reached between 44% and 88% of the maximum optical density (OD) across all treatments. After reaching peak concentrations, acetaldehyde decreased steadily.

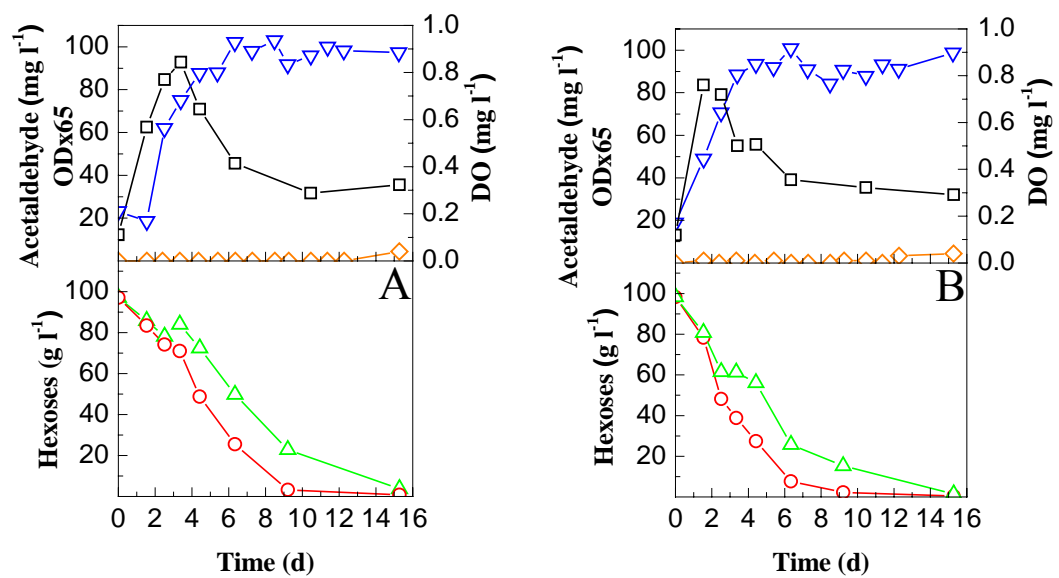


Figure 4.1 Alcoholic fermentation by *Saccharomyces cerevisiae* EC1118 in Sauvignon Blanc (A) and Gewürztraminer (B). Acetaldehyde (□); Growth as optical density at 650 nm (OD, ▽); dissolved oxygen (DO, ◇); glucose (○); fructose (△). Data shows mean values from duplicate incubations. Examples show control treatments with SO₂ and nutrients added, native pH value and a 20°C fermentation temperature.

Table 4.2 Effect of various vinification parameters on maximum and final acetaldehyde concentrations observed during alcoholic fermentation by two *S. cerevisiae* strains. Significant differences ($p < 0.05$) among average results of peak or final acetaldehyde concentrations (mg l^{-1}) observed in duplicate incubations denoted by letters. G, Gewürztraminer, SB, Sauvignon Blanc. Residual sugar measured 15 days post inoculation.

Treatment description	Strain EC1118	Strain DV10	SO ₂ Addition	pH Increase	12°C	20°C	Yeast Nutrient Addition	SB	G	Acetaldehyde Maximum*	Acetaldehyde Final*	Glucose/ Fructose Ratio at Max Acetaldehyde	Residual Sugar (g l^{-1})
No SO ₂ Addition	1					1	1	1		87±12 ^a	24±2 ^b	0.89±0.07	1.37±0.18
No Yeast Nutrient	1		1			1		1		97±1 ^a	35±1 ^a	0.91±0.01	5.06±0.38
pH 3.6	1		1	1		1	1	1		104±11 ^a	38±4 ^a	0.89±0.02	2.72±0.31
12°C	1		1		1		1	1		101±16 ^a	40±6 ^a	0.95±0.06	34.05±10.93
Fermentation Control for EC1118 in SB	1		1			1	1	1		93±9 ^a	36±0 ^a	0.85±0.04	4.51±0.78
No SO ₂ Addition	1					1	1		1	65±5 ^b	25±2 ^d	0.88±0.08	2.02±0.01
No Yeast Nutrient	1		1			1			1	92±2 ^a	37±2 ^a	0.83±0	2.91±0.84
pH 3.6	1		1	1		1	1		1	83±3 ^a	31±2 ^c	0.84±0.06	2.46±0.74
12°C	1		1		1		1		1	86±5 ^a	36±1 ^{ab}	0.84±0.04	16.85±3.32
Fermentation Control for EC1118 in G	1		1			1	1		1	86±9 ^a	32±0 ^{bc}	0.81±0.23	1.92±0.22
No SO ₂ Addition		1				1	1	1		107±4 ^b	26±0 ^d	0.90±0.01	2.50±0.25
No Yeast Nutrient		1	1			1		1		87±1 ^d	34±1 ^c	0.94±0.11	4.87±1.29
pH 3.6		1	1	1		1	1	1		119±5 ^a	38±0 ^b	0.92±0.05	2.80±0.19
12°C		1	1		1		1	1		100±2 ^c	49±1 ^a	0.89±0.07	38.09±1.62
Fermentation Control for DV10 in SB		1	1			1	1	1		101±1 ^c	35±0 ^c	1.02±0.01	3.90±0.86
No SO ₂ Addition		1				1	1		1	62±1 ^b	22±2 ^b	0.69±0.18	2.23±0.49

No Yeast Nutrient pH 3.6 12°C Fermentation Control for DV10 in G	1	1		1		1	78±1 ^a	33±2 ^a	0.90±0.11	1.80±0.30
	1	1	1	1	1	1	78±9 ^a	36±1 ^a	0.94±0.07	1.80±0.49
	1	1		1	1	1	82±2 ^a	36±3 ^a	0.82±0.01	9.91±0.23
	1	1		1	1	1	86±3 ^a	36±1 ^a	0.79±0.20	2.42±1.36

Figure 4.2 shows that the profile of the kinetics of acetaldehyde production and degradation was similar in all forty fermentations carried out. However, peak and final concentrations varied considerably and ranged from 62 to 119 mg l⁻¹ and 22 and 49 mg l⁻¹, respectively. Analysis of the relationship between maximum and final acetaldehyde values revealed a statistically significant positive linear relationship between the two variables (Figure 4.3), with Gewürztraminer fermentations having a higher correlation coefficient ($r^2 = 0.70$) than Sauvignon Blanc fermentations ($r^2 = 0.39$), which had slower fermentation rates.

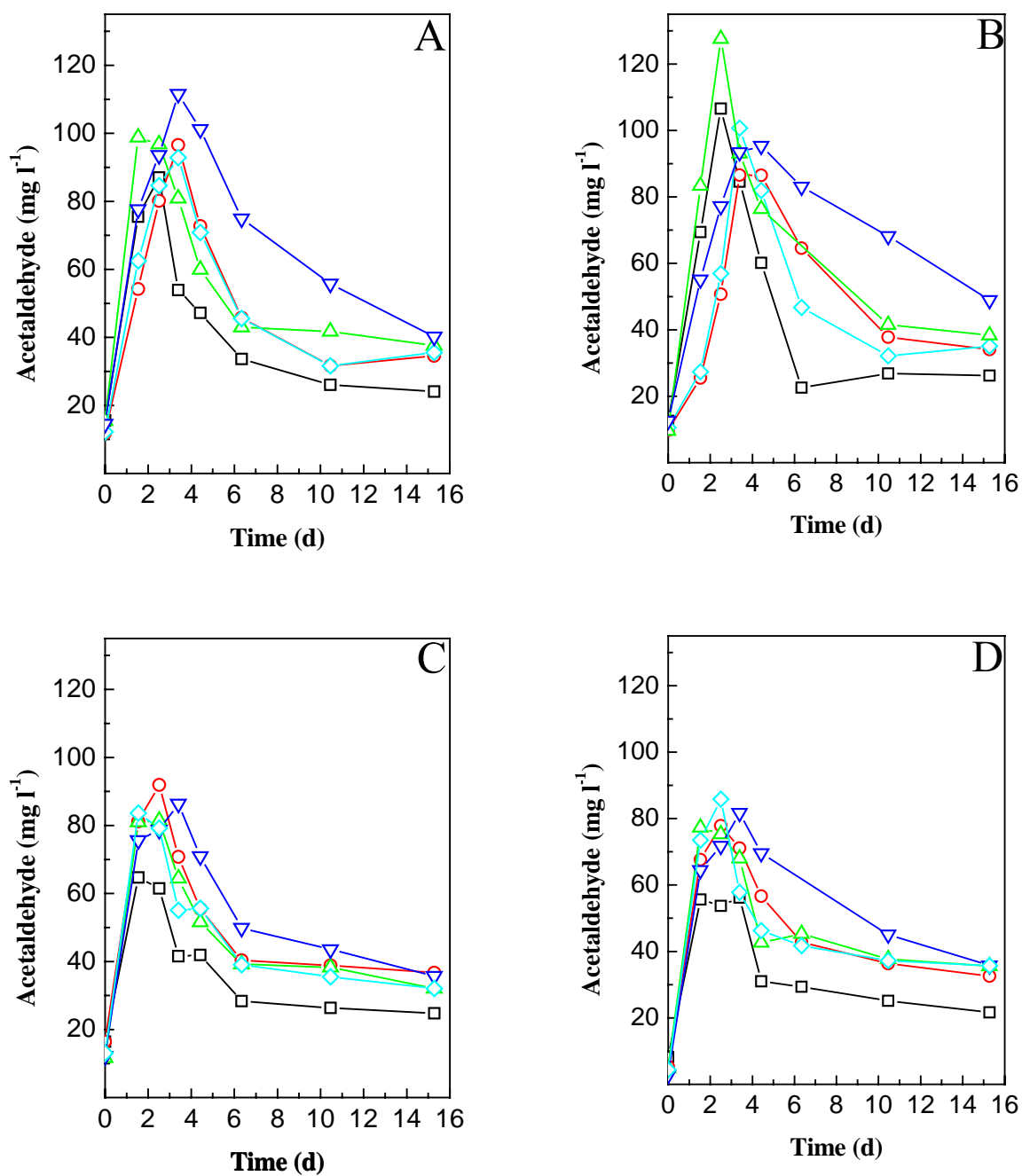


Figure 4.2 Acetaldehyde production and degradation kinetics in all treatments, Sauvignon Blanc (SB) with strain EC1118 (A); SB, DV10 (B); Gewürztraminer with strain EC1118 (C); G, DV10 (D). no SO₂ addition (□); no yeast nutrient (○); high pH (3.6, △); low temperature (12°C, ▽); control fermentation (30 mg l⁻¹ SO₂, 250 mg l⁻¹ of yeast nutrient, native pH, 20°C, ◇).

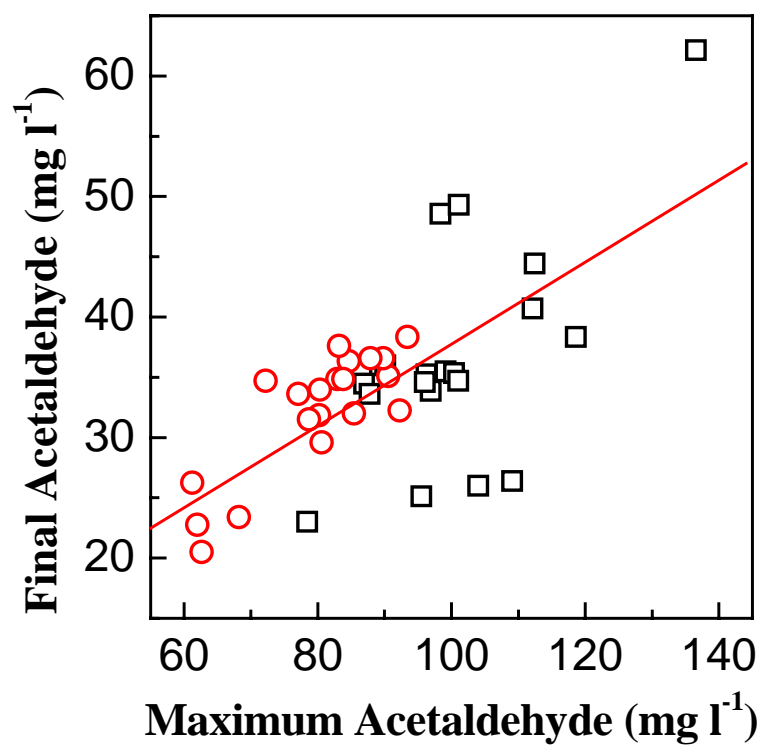


Figure 4.3 Correlation between maximum and corresponding final acetaldehyde concentrations ($r^2 = 0.46$, $Y = 3.78 \pm 5.42 + 0.34X \pm 0.06$) from 40 individual fermentations carried out with Sauvignon Blanc (□) and Gewürztraminer (○). ANOVA F-test statistic 32.7 ($p < 0.001$).

Using least squares regression analysis, individual enological parameters and interaction effects were tested for their ability to significantly affect peak and final acetaldehyde levels. While some of the six investigated factors were found to have no statistically significant effect on acetaldehyde kinetics associated parameters, others were of great relevance for peak and/or final acetaldehyde concentrations, either alone or in combination (Table 4.3). Addition of SO₂ to the must led to higher peak and final acetaldehyde levels across treatments (average increase of 11 and 12 mg l⁻¹ in peak and final SO₂ levels from a 30 mg l⁻¹ SO₂ addition, respectively), which were particularly noticeable in the case of peak acetaldehyde values in Gewürztraminer fermentations (average increase of 63±3 and 84±1 mg l⁻¹ without and with SO₂, respectively). Across treatments, Sauvignon Blanc led to higher final and, especially, peak acetaldehyde levels (Table 4.3), 98±2 mg l⁻¹ for combined Sauvignon Blanc fermentations compared with 80±2 mg l⁻¹ in combined Gewürztraminer fermentations). In Sauvignon Blanc fermentations, but not Gewürztraminer, the pH significantly affected peak and acetaldehyde levels. For yeast strain EC1118, omission of nutrient led to significantly higher peak, but not final acetaldehyde levels across treatments (88 and 94 mg l⁻¹ of acetaldehyde in fermentations without and with nutrient, respectively). Temperature was observed to significantly affect the final but not maximum acetaldehyde concentrations. All fermentations carried out at 12°C had higher mean final acetaldehyde residues (increase of 40 mg l⁻¹) with respect to their 20°C controls (32 mg l⁻¹) and this effect was particularly pronounced in the slower Sauvignon Blanc fermentations (45 and 32 mg l⁻¹ at 12°C and 20°C, respectively).

Table 4.3 Analysis of the effect of six enological parameters on the maximum and final acetaldehyde concentrations by standard least squares regression analysis. P-values indicate statistically significant difference between two chosen parameters for single effects (i.e. one parameter) and statistical difference between groups of 2 parameters for interaction effects.

Parameter	Maximum			Final		
	Factor 1	Factor 2	p-value	Factor 1	Factor 2	p-value
SO ₂ Added (mg l ⁻¹)	0	30	0.0023 [†]	0	30	<0.0001 [†]
Mean (mg l ⁻¹)	80 ± 5	91 ± 2		24 ± 1	36 ± 1	
Grape Variety	SB	G	<0.0001 [†]	SB	G	0.0190 [†]
Mean (mg l ⁻¹)	98 ± 2	80 ± 2		35 ± 1	32 ± 1	
Grape Variety*pH	SB*3.1	SB*3.6	0.0109 [†]	SB*3.1	SB*3.6	0.1440
Mean (mg l ⁻¹)	96 ± 2	109 ± 5		32 ± 1	33 ± 2	
Grape Variety*SO ₂ (30 mg l ⁻¹)	G*No SO ₂	G*SO ₂	0.0023 [†]	G*No SO ₂	G*SO ₂	0.8466
Mean (mg l ⁻¹)	63 ± 3	84 ± 1		25 ± 2	38 ± 1	
Grape Variety*Yeast	SB*DV10	G*DV10	0.0151 [†]	SB*DV10	G*DV10	0.2086
Mean (mg l ⁻¹)	101 ± 3	77 ± 3		36 ± 2	32 ± 2	
Yeast*Yeast Nutrient	EC1118* Nutrient	EC1118* No Nutrient	0.0278 [†]	EC1118* Nutrient	EC1118* No Nutrient	0.1370
Mean (mg l ⁻¹)	88 ± 3	94 ± 6		36 ± 3	33 ± 1	
Temperature (°C)	20	12	0.7963	20	12	0.0002 [†]
Mean (mg l ⁻¹)	88 ± 2	92 ± 5		32 ± 1	40 ± 2	
Grape*Temperature (°C)	SB*20	SB*12	0.3938	SB*20	SB*12	0.0079 [†]
Mean (mg l ⁻¹)	98 ± 3	100 ± 5		33 ± 1	45 ± 3	

[†] Statistically significant finding (p < 0.05). Means ± SE.

Comparison of acetaldehyde and hexose kinetics led to several observations. In Gewürztraminer treatments, acetaldehyde peaks occurred after an average degradation of 31% of combined glucose and fructose while in Sauvignon Blanc treatments only 16% had been degraded, a difference that proved to be significant ($p < 0.0001$). Furthermore, a comparison of the initial kinetics revealed that the acetaldehyde production yield coefficients were higher in Sauvignon Blanc fermentations compared to Gewürztraminer (3.02 and 1.20 mg acetaldehyde produced per gram of hexose degraded, respectively).

A common observation across all treatments was that the time point of peak acetaldehyde concentrations coincided with the divergence of glucose and fructose degradation rates, as exemplified by the experiments shown in Figure 4.1. Further analysis (Table 4.2) revealed that at the time point of peak acetaldehyde levels, glucose to fructose ratios were relatively uniform across treatments with values ranging between 0.69 and 1.02 (mean 0.88 ± 0.07). A graphical comparison of the time points of hexose divergence defined as a glucose to fructose ratio of 0.9 and peak acetaldehyde levels is shown in Figure 4.4.

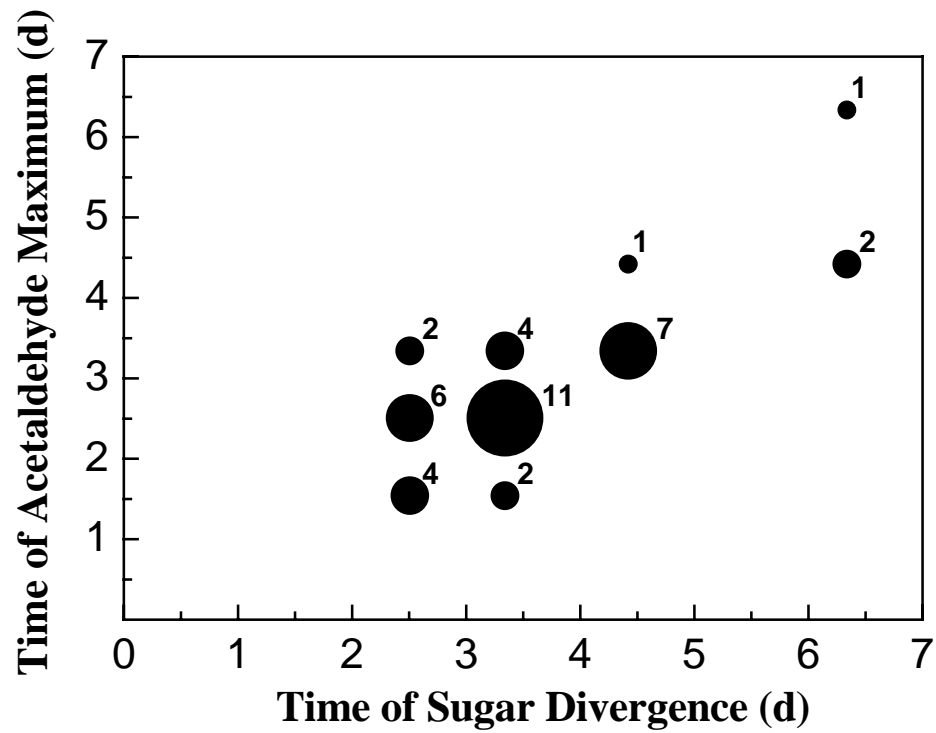


Figure 4.4 Time of sugar divergence compared to the time of peak acetaldehyde concentrations in forty alcoholic fermentations with *S. cerevisiae*. Size of symbols and adjacent number represent number of data points at identical coordinates.

4.5. Discussion

Acetaldehyde is the terminal electron acceptor in the alcoholic fermentations (AF) of *Saccharomyces cerevisiae*. Quantitatively, it is the most important carbonyl by-product of the AF, and has relevance for ethanol production yields as well as product stabilization and toxicology.

This study investigated acetaldehyde kinetics during AF as a factor of six different parameters. Two white grape musts and two commercially available strains of *S. cerevisiae* were used while varying, SO₂ and complex nutrition additions, must pH and fermentation temperature within usual enological ranges. The yeast strains were selected for their widespread utilization in the wine industry, tolerance to wide temperature and alcohol ranges and low nutrient requirements (Barbosa et al., 2009; Pizarro et al., 2008; Wang et al., 2003).

In complex systems containing transition metals and polyphenols, such as wine, involuntary oxidation of ethanol leads to formation of acetaldehyde (Danilewicz, 2003; Danilewicz, 2007) but the prevention and control of involuntary oxidation has not been reported in most published studies on microbial formation and degradation of acetaldehyde (Herraiz et al., 1989; Ibeas et al., 1997; Millán and Ortega, 1988; Romano et al., 1994; Romano et al., 1997). In this work, fermentation containers were kept full, air ingress was limited with suitable air locks and samples were taken under a constant stream of nitrogen gas. Continuous measurement of dissolved oxygen using a sensitive and non-invasive dissolved oxygen meter confirmed the suitability of this method.

All cultivations carried out in this study displayed similar acetaldehyde kinetics confirming previous work on this metabolite (Cherai et al., 2010; Millán and Ortega, 1988; Osborne et al., 2006; Weeks, 1969). However, in contrast with a recent study by Cherai et al. (2010), a statistically significant linear relationship between peak and final acetaldehyde levels was found across all vinifications in this study. This relationship was not as strong if the course of the fermentations was sluggish, such as in the case of Sauvignon Blanc, or if the fermentation temperatures were low indicating that a poor relationship between peak and final values may be caused especially by lower reutilization of acetaldehyde in the final fermentation phase.

Among the enological parameters tested, the utilization of SO₂ was assumed to have a high potential to affect acetaldehyde concentrations during AF. Acetaldehyde levels are primarily controlled by pyruvate decarboxylase (PDC) production and alcohol dehydrogenase (ADH) degradation (Bakker et al., 2000; Berowska et al., 2009). The strength of the SO₂-acetaldehyde bond is very strong ($K_d = 2.06 \times 10^{-6}$) (Burroughs and Sparks, 1973) and hence, SO₂ scavenges the terminal electron acceptor of the AF. A recent study by Park and Hwang (2008) demonstrated that SO₂ induced transcription of enzymes involved in carbohydrate metabolism, particularly PDC, and highlighted the role of acetaldehyde in detoxifying SO₂, which can negatively effect energy metabolism in *S. cerevisiae*.

Theoretically, 30 mg l⁻¹ SO₂ could bind approximately 20 mg l⁻¹ of acetaldehyde. However, in this study peak and final acetaldehyde concentrations rose by 11 and 12 mg l⁻¹ after sulfite addition, respectively. Accordingly, 366 µg of acetaldehyde were formed per mg of SO₂ added to the must. These values require confirmation with other

yeast and musts and will be of practical significance in helping producers assess the impact of SO₂ additions on bound SO₂ levels, which are of increasing significance considering reductions in total permitted SO₂ levels in some legislations (Anonymous, 2009).

Multifactorial analysis of the data obtained allowed to establish which other enological factors were of relevance for acetaldehyde kinetics. Across all experiments, cool fermentation temperatures (12°C versus 20°C) led to higher acetaldehyde residues even though there was no statistically significant effect on peak acetaldehyde levels. The high acetaldehyde residue could be explained by the lower rate of acetaldehyde reutilization in the second half of the fermentation and was associated with higher sugar residues. These observations are confirmed by Sohrabvandi et al. (2009) who showed that that final acetaldehyde levels could be reduced by *S. cerevisiae* using higher fermentation temperatures (24°C, 13 mg l⁻¹; 12°C, 24 mg l⁻¹). However, the results are inconsistent with those obtained by Romano et al. (1994) who reported that 30°C fermentations produced more acetaldehyde than those at 12°C, 18°C or 24°C and Torija et al. (2003) who observed that 20°C fermentations led to 90 mg l⁻¹ final acetaldehyde concentrations while 50 mg l⁻¹ and 20 mg l⁻¹ were obtained at 15°C and 35°C, respectively. It is important to note that none of these studies reported anaerobic handling of cultivations and samples, or dissolved oxygen measurements. The results obtained here are relevant in suggesting that yeast activity during the latter phases of the fermentation and during yeast lees ageing periods are relevant in reducing acetaldehyde residues.

Other parameters, individually or in combination, only had small effects on acetaldehyde residues. However, important effects on peak acetaldehyde levels could be established for parameters that characterized the medium composition, i.e. nutrient addition, the pH, and especially the grape variety. Gewürztraminer overall led to significantly lower final acetaldehyde concentrations despite of Brix, pH and titratable acidity levels that were similar to those of the Sauvignon Blanc. The major observable difference between the two musts was the native YAN content, which was 28% higher in Gewürztraminer. Lemperle (1994) correlated higher final acetaldehyde levels to musts with low nitrogen concentrations, due to increased SO₂ production in selected yeast strains. However, the lack of overall difference among treatments with and without yeast nutrient added found in this study, makes it unlikely that the availability of YAN may have been solely responsible for the varietal effect observed. Jarvis and Lea (2000) found a correlation between low thiamine and pantothenate levels and higher final acetaldehyde concentrations. Low zinc concentrations have also been implicated in increased final acetaldehyde levels (Bird et al., 2006). While there are no studies providing comprehensive compositional data of commercial yeast nutrient products, the results in this study suggest that yeast acetaldehyde kinetics may be susceptible to micronutrient deficiencies that may not be adequately addressed by the supplementation with the complex nutrients used here.

Further consideration of the kinetics of acetaldehyde and sugar metabolism showed an association between the time of peak acetaldehyde levels and the divergence in the sugar catabolism, i.e. the time point where the glucose degradation rate greatly increased over the rate of fructose degradation. High discrepancies of glucose and

fructose concentrations have been associated with sluggish and stuck fermentation (Bisson, 1999). A statistically significant correlation could not be established between both parameters in this study since the sampling frequency did not allow to determine the time of acetaldehyde peaks more precisely. However, this novel observation warrants further investigation about the possible relationship between acetaldehyde metabolism, sugar degradation and fermentation success. In this context, it is noteworthy that acetaldehyde has been shown to cause apoptosis in some model cell systems (Menegola et al., 2001). While there is some research concerning the factors that mediate apoptosis of yeast in enological fermentations, the role of acetaldehyde has not been considered yet (Gerhards et al., 2010).

4.6. Conclusions

This study considered the effect of various enological parameters on acetaldehyde kinetics during alcoholic fermentations. Regardless of the treatment, acetaldehyde kinetics were uniform across fermentations with an initial increase to a peak value, followed by reutilization. Several parameters were found to affect peak and/or final acetaldehyde concentrations either individually or in combination. Except for sluggish fermentations, and those conducted at low temperatures, the peak acetaldehyde concentration was well correlated with the residual levels after 15 days of fermentation. The results allow to estimate acetaldehyde increases caused by SO₂ addition and indicate that the ability of the must composition to suffice yeast nutritional requirements play a role in mediating acetaldehyde dynamics. The final fermentation phase was shown to be significant in reducing acetaldehyde residues. A novel relationship was found between the occurrence of the peak acetaldehyde

concentrations and the divergence of glucose and fructose degradation rates, which requires further consideration.

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CHAPTER 5: METABOLISM OF SO₂ BINDING COMPOUNDS BY *OENOCOCCUS OENI* DURING AND AFTER MALOLACTIC FERMENTATION IN WHITE WINE.

5.1. Abstract

Sulfur dioxide (SO₂) is the key additive for the preservation of wines. Carbonyls in wine can bind to SO₂ and decrease its efficacy, resulting in higher total SO₂ requirements. Increased consumer demand for low sulfite and organic wines pose production challenges if SO₂ binders have not been properly managed during vinification. Malolactic fermentation (MLF) has been known to reduce bound SO₂ levels but detailed time course studies are not available. In this work, the kinetics of major SO₂ binding compounds and malic acid were followed during malolactic fermentation (MLF) in wine with 12 commercially available strains of *Oenococcus oeni*. Pyruvic acid, acetaldehyde and α -ketoglutaric acid were degraded to various degrees by *O. oeni*, but galacturonic acid was not. At the time of malic acid depletion, percent degradation of pyruvate, α -ketoglutaric acid and acetaldehyde was 49%, 14% and 30%, respectively. During MLF, the decrease in average bound SO₂ levels, as calculated from carbonyl metabolism, was 22%. The largest reduction in wine carbonyl content occurred in the week after completion of MLF and was 53% (107 mg l⁻¹ to 34 mg l⁻¹) calculated as bound SO₂. Prolonged activity of bacteria in the wines (up to 3 weeks post malic acid depletion) resulted only in reduced additional reductions in bound SO₂ levels.

The results suggest that microbiological wine stabilization one week after malic acid depletion is an effective strategy for maximum removal of SO₂ binders while reducing

the risk of possible post-ML spoilage by *O. oeni* leading to the production of acetic acid and biogenic amines.

5.2. Introduction

Sulfur dioxide (SO₂) is an important preservative commonly used in winemaking and the production of other foods (Doyle and Beuchat, 2007). Its anti-microbial (Carreté et al., 2002), anti-oxidant (Danilewicz, 2003) and anti-enzymatic (Main and Morris, 1991; Wedzicha et al., 1991) functionality at low concentrations makes SO₂ an ideal and cost effective food stabilizer. Despite its value and potency, a majority of consumers view sulfite additions to wine as unnatural and unhealthy (Stolz and Schmid, 2008). These apprehensions about SO₂ utilization may stem from historical sulfite abuses in some grocery stores and restaurants (Martin et al., 1986) and sulfite warning labels on wine bottles. The US FDA estimates that 1% of the U.S. population show an increased degree of sensitivity to sulfites (Papazian, 1996). Asthmatics appear to be especially susceptible, with estimates that up to 5% may risk adverse reactions upon sulfite exposure (Snelten and Schaafsma, 1992; Vally et al., 1999). Minimally processed foods and wines with little or no sulfites have been increasingly popular with consumers (Azabagaoglu et al., 2007). In the U.S., sales of certified organic foods have increased from \$78 million in 1980 to approximately \$6 billion in 2000, with an average annual increase of 24% during the 1990s (Hughner et al., 2007). The organic wine sector has also followed this trend, and in 2005 the world organic wine market grew by 10 - 15% (Richter and Padel, 2007).

To meet increasing consumer demands, winemakers are challenged to restrict or even eliminate sulfites during vinification, while maintaining high product quality. An

important strategy has been to decrease concentrations of carbonyl or keto compounds that bind with SO₂, thus decreasing its preservative activity compared to free SO₂ (Rankine, 1968; Ribéreau-Gayon et al., 1998a). Acetaldehyde is an important SO₂ binding compound found in wine, primarily due to the low dissociation constant of its sulfonate ($K_d = 1.5 \times 10^{-6}$) (Ribéreau-Gayon et al., 1998a). Other SO₂ binding compounds including pyruvate, α -ketoglutaric and galacturonic acids may also have a significant effect on bound SO₂ levels (Ribéreau-Gayon et al., 1998a).

Previous studies have recognized the importance of malolactic fermentation (MLF) to reduce the pool of SO₂ binders in wine (Flamini et al., 2002; Radler, 1986; Zaunmuller et al., 2006), especially acetaldehyde (Osborne et al., 2000). The aim of this work was to provide the comprehensive analysis of the kinetics of acetaldehyde, pyruvate, α -ketoglutaric acid and galacturonic acid concentrations during MLF with 12 commercial strains of heterofermentative *Oenococcus oeni* in wine. This thorough analysis of SO₂ binders during and after malic acid metabolism, with concurrent modeling of bound SO₂ levels, offers new insights for sulfite management during vinification.

5.3. Materials and Methods

5.3.1. Microorganisms and chemicals

Saccharomyces cerevisiae strain CY3079 and 12 commercial *Oenococcus oeni* strains were provided by Lallemant Inc. (Montréal, Canada) and stored according to the manufacturer's recommendations. All chemicals, unless cited otherwise, were of analytical grade from Thermo Fisher Scientific (Fairlawn, NJ).

5.3.2. Grape must and general inoculation procedures

A flash pasteurized German Riesling (Kamil EX-IM, Canada) must was used as medium for all fermentations in this study. The analytical profile of the must and the wine after alcoholic fermentation is summarized in Table 4.1. Yeast inoculation was conducted in accordance with supplier's recommendations; 250 mg l⁻¹ of each yeast (CY3079) and yeast hydration nutrient (GoFerm, Lallemand, Canada) were each added to the grape must. Alcoholic fermentation was completed in 2.0 U.S. gallon jugs with air locks to dryness (<5.0 g l⁻¹ sugars). Bentonite (2.0 g l⁻¹ Ca-Granulat, Erbslöh Geisenheim, Germany) was added after the completion of alcoholic fermentation to aid in yeast settling and clarification. Wines were then cold stabilized for 5 days at 4°C and racked in preparation for sterile filtration using nylon membrane filters (0.45 µm, Millipore, Ireland). 200 ml aliquots of Riesling wine were aseptically transferred into previously sterilized 250 ml glass bottles for inoculation with *Oenococcus oeni*. *O. oeni* strains were pre-grown in sterile MRS media (BD, Franklin Lakes, NJ) (pH 4.5) until stationary phase, and then centrifuged at 2000 g for 10 minutes to concentrate biomass (~1.0x10⁸ CFU ml⁻¹). Cells were washed using a sodium hydrogen tartrate buffer containing 7.5 g l⁻¹ tartaric acid, 495 mg l⁻¹ MgSO₄, 345 mg l⁻¹ MnSO₄·5H₂O and 0.005% (w/v) Tween 80 adjusted to pH 4.5 with 5N NaOH. *O. oeni* suspensions thus obtained were then inoculated into each container at a rate of 1% v/v and held at room temperature until completion of MLF.

Table 5.1 Analytical profile of Riesling grape must and wine.

Must Parameters				Wine Parameters				
Soluble Solids (Brix)	pH	Titratable Acidity (Tartaric Acid g l ⁻¹)	Total YAN (mg l ⁻¹)	Ethanol (%v/v)	Concentration of SO ₂ binding compounds before MLF (mg l ⁻¹)			
					AcHO	Pyr	α -KG	GA
20.6	3.4	7.22	106.22	10.96	84.6±1.0	11.7±0.5	24.6±0.3	612.4±17.2

AcHO (acetaldehyde), Pyr (pyruvate), α -KG (α -ketoglutaric acid), GA (galacturonic acid)

5.3.3. Sampling

During MLF, samples were taken regularly and immediately frozen (-18°C) for future analysis. During sampling, food grade nitrogen (Airgas, NY) was used to flush the headspace of fermentation bottles and limit oxygen ingress.

5.3.4. Analytical methods and statistical analysis

Glucose, fructose and malic acid were measured enzymatically using a commercial test kit (Megazyme, Ireland). Titratable acidity was determined by titration of must with 0.1M NaOH, using phenolphthalein as an indicator. Total yeast assimilable nitrogen (YAN) was calculated as the sum of primary amino acids and total ammonia. Primary amino acid content was quantified using the NOPA method defined by Dukes & Butzke (1998), while ammonia was quantified using an ion selective electrode (Ammonia ISE electrode, Cole Palmer, Vernon Hills, IL) according to the method of McWilliams & Ough (1974). Alcohol content (%v/v) was quantified by near infrared spectroscopy (Alcolyzer, Anton Paar, Germany).

5.3.5. Chemicals and preparation of reagents for SO₂ binder analysis

2,4-dinitrophenylhydrazine (DNPH) had a purity of 97% (as dry weight) and contained 30-40% water to reduce the explosion hazard. ASTM Class I water was prepared using a water purification system (Arium 611UV, Sartorius, Germany) which was used as a solvent for all reagents and UHPLC solvent A (Table 5.2).

The derivatizing reagent was prepared by dissolving DNPH in acetonitrile adjusted to pH 1.5 with perchloric acid to obtain an 11 mM DNPH solution. For sample pre-treatments, an aqueous solution of 86 mM EDTA in 1 M sodium hydroxide was

prepared and degassed (Aquasonic Model 150D, VWR International, PA) for 20 minutes. Solutions were stored up to one week at 4°C.

Table 5.2 Gradient program for the chromatographic separation of DNPH derivatized SO₂ binding compounds using a Phenomenex Kinetex C18 column (100 x 3.0 mm).

Time (min)	Solvent A	Solvent B
0.00	85	15
4.25	82	18
4.75	75	25
6.50	62	38
12.00	40	60
12.50	10	90
13.25	85	15

5.3.6. Derivatization procedure

Derivatizations were conducted in 2.0 ml glass HPLC vials with Teflon caps (National Scientific, TN). For sulfonate hydrolysis, 200 μ l of sodium hydroxide/EDTA solution were added to the vial, followed by 100 μ l of sample wine and mixing. After 10 minutes, 200 μ l of 1M perchloric acid was added followed by 800 μ l of the derivatizing reagent. After mixing, the solution was allowed to react for exactly 30 hours at $30.0 \pm 0.1^\circ\text{C}$ and then promptly cooled to 4°C until analysis. Derivatized samples thus prepared were stable for up to five days.

5.3.7. HPLC Analysis

An ultra high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AD XR pumping unit, a DGU-20A₃ degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven, and a SPD-20A UV/VIS detector were used for separation and analysis of DNPH derivatized wine carbonyls. Data acquisition and analysis was performed with the LCSolution software (1.23). Solvent A consisted of water acidified to $\text{pH } 2.50 \pm 0.01$ using perchloric acid. Solvent B was HPLC grade acetonitrile. All solvents were filtered prior to utilization (0.22 μm , nylon, Millipore, Ireland). Samples were held at 4°C in the autosampler and 5.0 μ l of sample injected directly. Separation occurred on a Phenomenex Kinetex C18 stationary phase (100 x 3.0 mm) with 2.6 μm particle size held at 37°C with a flow rate of 0.75 ml min^{-1} . The analytes were quantified at 365 nm using external calibration standards with linear regression analysis. The separation gradient is shown in Table 5.2.

5.3.8. Statistical analysis

Student's t-tests were conducted with JMP 7.0 (SAS, North Carolina) to determine statistical significance between sample populations at the 0.05 confidence level.

5.4. Results

Complete malic acid metabolism was achieved during all malolactic fermentations using 12 commercial strains of *O. oeni*. Figure 5.1 illustrates the concentrations of malic acid and four SO₂ binding compounds during MLF in two exemplary fermentations. The start of malic acid degradation and the time point of its completion was strain dependant. The average duration for malic acid depletion was 25 days after inoculation, with minima and maxima of 16.9 and 43.8 days (R1108 and R1032, respectively, Table 5.3).

With the exception of galacturonic acid, the concentrations of all other SO₂ binders studied were strongly affected following inoculation with *O. oeni*. Acetaldehyde concentrations decreased rapidly during MLF and followed the metabolism of malic acid with slight delay. A rapid initial acetaldehyde degradation phase could be distinguished from a second slower phase (Figure 5.1). Across all strains, the rapid metabolism of acetaldehyde was completed between 2.0 and 8.4 days after malic acid depletion with an average delay of 4.9 days (Table 5.3). In all fermentations, acetaldehyde degradation continued after depletion of malic acid. From an average of 30% degradation at the time of malic acid depletion, 75% and 87% degradation were observed at the end of the rapid acetaldehyde metabolism phase, and three weeks after malic acid depletion, respectively (Table 5.4).

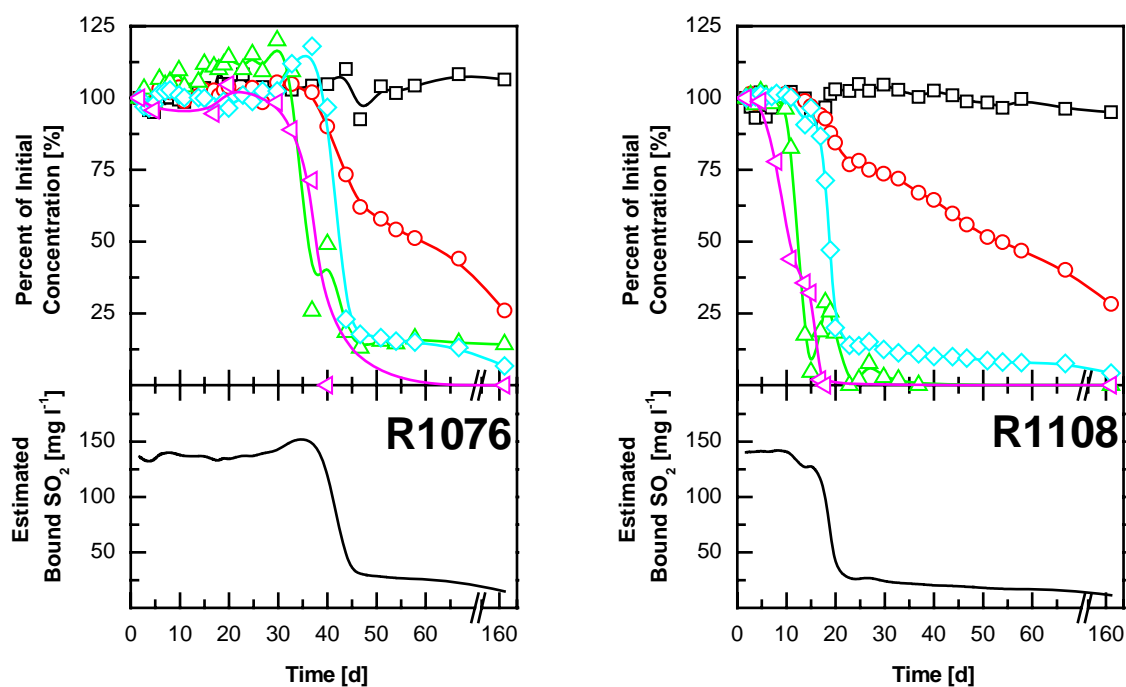


Figure 5.1 Kinetics of malic acid (\blacktriangleleft), galacturonic acid (\square), α -ketoglutaric acid (\circ), pyruvic acid (\blacktriangle) and acetaldehyde (\blacklozenge) during MLF in Riesling wine using *O. oeni* strains R1076 and R1108. Estimated bound SO_2 levels were calculated based on 30 mg l^{-1} free SO_2 .

Table 5.3 Comparison between the time point of malic acid depletion and the end of the rapid metabolism phase of the carbonyls acetaldehyde and pyruvic acid during MLF in Riesling by 12 strains of *O. oeni*. Mean values \pm SE are given.

Strain	Time of malic acid depletion [d]	Time until end of rapid carbonyl metabolism phase [d]		Time difference between malic acid depletion and end of rapid carbonyl metabolism phase [d]	
		AcHO	Pyr	AcHO	Pyr
R1032	43.8 \pm 0.0	48.8 \pm 2.1	38.4 \pm 1.6	5.0 \pm 2.1	-5.36 \pm 1.6
R1054	24.8 \pm 0.0	28.3 \pm 1.5	23.4 \pm 0.6	3.5 \pm 1.5	-1.35 \pm 0.6
R1075	43.8 \pm 0.0	48.8 \pm 2.1	38.4 \pm 1.6	5.0 \pm 2.1	-5.36 \pm 1.6
R1076	38.4 \pm 1.6	41.9 \pm 1.9	36.8 \pm 0.0	3.4 \pm 1.9	-1.64 \pm 1.6
R1077	22.8 \pm 0.0	24.8 \pm 0.0	17.4 \pm 0.5	2.0 \pm 0.0	-5.4 \pm 0.5
R1098	18.4 \pm 0.0	26.8 \pm 0.0	17.4 \pm 0.5	8.4 \pm 0.0	-1.0 \pm 0.5
R1101	19.9 \pm 0.5	28.3 \pm 1.5	17.4 \pm 0.5	8.4 \pm 1.5	-2.5 \pm 0.5
R1105	16.9 \pm 0.0	19.9 \pm 0.0	14.4 \pm 0.5	3.0 \pm 0.0	-2.5 \pm 0.6
R1106	17.4 \pm 0.0	24.8 \pm 0.0	14.4 \pm 0.6	7.4 \pm 0.0	-3.0 \pm 0.6
R1108	16.9 \pm 0.5	22.3 \pm 2.4	14.4 \pm 0.6	5.4 \pm 2.4	-2.5 \pm 0.6
R1118	16.9 \pm 0.0	19.9 \pm 0.0	14.4 \pm 0.6	3.0 \pm 0.0	-2.5 \pm 0.6
R1124	19.9 \pm 0.0	24.8 \pm 0.0	17.4 \pm 0.5	4.9 \pm 0.0	-2.5 \pm 0.5
Mean	25.0\pm2.1	29.9\pm2.1	22.0\pm7.0	4.9\pm0.6	-3.0\pm0.4
Range	16.9 – 43.8	19.9 – 48.8	14.4 – 38.4	2.0 – 8.4	-5.36 – -1.0

AcHO (acetaldehyde), Pyr (pyruvic acid)

Table 5.4 Percent degradation of SO₂ binding compounds displayed at the time of malic acid depletion, at the end of the rapid carbonyl degradation phase and 3 weeks after MLF completion. Mean values are given \pm SE.

Strain	Percent degradation									
	At time of malic acid depletion [%]				At end of rapid metabolism phase [%]		3-weeks post malic acid depletion [%]			
	AcHO	Pyr	α -KG	GA	AcHO	Pyr	AcHO	Pyr	α -KG	GA
R1032	28.7 \pm 11.3	68.1 \pm 2.9	20.9 \pm 1.4	0.0 \pm 0.0	58.8 \pm 18.9	97.0 \pm 3.1	82.7 \pm 1.5	100.0 \pm 0.0	54.7 \pm 4.3	0.0 \pm 0.0
R1054	31.6 \pm 12.4	70.8 \pm 2.2	24.5 \pm 3.5	0.0 \pm 0.0	60.9 \pm 17.0	83.4 \pm 0.3	92.3 \pm 0.1	83.4 \pm 0.5	56.3 \pm 1.5	0.0 \pm 0.0
R1075	24.3 \pm 0.0	63.7 \pm 4.3	21.2 \pm 2.2	0.0 \pm 0.0	78.0 \pm 2.7	81.6 \pm 1.9	82.8 \pm 1.4	87.5 \pm 0.1	71.8 \pm 2.8	0.0 \pm 0.0
R1076	13.3 \pm 10.0	50.9 \pm 1.1	9.9 \pm 2.1	0.0 \pm 0.0	79.7 \pm 2.6	84.3 \pm 2.7	84.1 \pm 0.6	84.6 \pm 0.8	52.4 \pm 3.5	0.0 \pm 0.0
R1077	48.9 \pm 15.6	54.7 \pm 3.3	15.7 \pm 3.7	0.0 \pm 0.0	82.1 \pm 1.2	80.4 \pm 1.9	88.8 \pm 0.5	86.5 \pm 0.7	50.2 \pm 3.4	0.0 \pm 0.0
R1098	18.8 \pm 4.8	8.7 \pm 1.7	12.1 \pm 1.1	0.0 \pm 0.0	79.2 \pm 7.0	79.8 \pm 1.2	91.8 \pm 0.7	91.7 \pm 0.4	53.4 \pm 1.6	0.0 \pm 0.0
R1101	16.4 \pm 13.1	49.6 \pm 5.4	8.9 \pm 2.1	0.0 \pm 0.0	68.3 \pm 12.9	77.9 \pm 2.4	91.4 \pm 0.3	88.9 \pm 1.5	35.2 \pm 1.9	0.0 \pm 0.0
R1105	24.6 \pm 8.2	-20.1 \pm 5.1	8.1 \pm 2.1	0.0 \pm 0.0	68.5 \pm 10.4	66.5 \pm 0.9	86.9 \pm 0.2	67.8 \pm 0.1	47.3 \pm 1.9	0.0 \pm 0.0
R1106	29.1 \pm 6.2	42.4 \pm 4.6	12.9 \pm 1.9	0.0 \pm 0.0	77.9 \pm 6.1	71.2 \pm 0.4	85.8 \pm 0.2	71.7 \pm 0.3	52.2 \pm 1.8	0.0 \pm 0.0
R1108	21.0 \pm 7.7	81.3 \pm 2.3	5.9 \pm 3.1	0.0 \pm 0.0	83.0 \pm 3.0	95.2 \pm 2.9	83.2 \pm 1.3	100.0 \pm 0.0	34.3 \pm 1.3	0.0 \pm 0.0
R1118	43.9 \pm 10.1	59.4 \pm 2.6	10.6 \pm 2.6	0.0 \pm 0.0	79.9 \pm 1.6	67.4 \pm 0.4	88.3 \pm 0.4	78.6 \pm 1.6	28.1 \pm 2.3	0.0 \pm 0.0
R1124	64.5 \pm 16.7	56.9 \pm 0.4	19.9 \pm 0.9	0.0 \pm 0.0	82.2 \pm 1.0	76.9 \pm 0.7	87.7 \pm 0.2	80.9 \pm 0.3	43.3 \pm 1.2	0.0 \pm 0.0
Mean	30.4\pm3.7	48.9\pm5.7	14.2\pm1.3	0.0\pm0.0	74.9\pm2.5	81.1\pm1.9	87.1\pm1.0	85.1\pm4.7	48.2\pm2.0	0.0\pm0.0
Range	13.3 – 64.5	-20.1 – 81.3	5.9 – 24.5	N/A	58.8 – 83.0	66.5 – 95.2	82.8 – 92.3	67.8 – 100.0	28.1 – 71.8	N/A

AcHO (acetaldehyde), Pyr (pyruvate), α -KG (α -ketoglutaric acid), GA (galacturonic acid)

A clear distinction of an initial rapid, and a subsequent slower degradation phase was also observed for pyruvic acid. However, a transient increase of pyruvic acid concentrations that occurred towards the end of malic acid metabolism could be observed in most fermentations studied (Figure 5.1). In contrast to acetaldehyde, the rapid degradation of pyruvic acid was not delayed in comparison with the metabolism of malic acid. On average, the end of the rapid pyruvic acid degradation was found to occur 3 days before the depletion of malic acid (Table 5.3). Across all strains, degradation of pyruvic acid increased from 49% at the time of malic acid depletion to 85% three weeks post-MLF (Table 5.4).

The onset of the α -ketoglutaric acid degradation was similar to the start of acetaldehyde degradation, but the degradation rates were lower thereafter (Figure 5.1). At the time of malic acid depletion 6% - 25% ($\bar{O}=14.2$) of α -ketoglutaric acid had been depleted increasing to 28%–72% ($\bar{O}=48.2$) three weeks post-MLF (Table 5.4).

For any given time point, the concentrations of SO₂ binders were used to estimate the total level of bound SO₂ if the wines were to be stabilized. For this calculation, the dissociation constants reported by Ribéreau-Gayon et al (1998b) were used, and the wine was assumed to have 30 mg l⁻¹ of free SO₂ and no other SO₂ binders. Acetaldehyde was found to be the most influential SO₂ binding compound for the overall calculated level of bound SO₂ (Figure 5.1). Metabolism of SO₂ binding compounds by *O. oeni* concomitantly reduced calculated mean bound SO₂ levels by 22% at the time-point of malic acid depletion. Wine contact with the bacteria after malic acid depletion led to a further and steady decrease in calculated bound SO₂ concentrations. The mean bound SO₂ level reductions were 75%, 81% and 83%

measured at one, two and three weeks after malic acid depletion, respectively (Table 5.5).

Table 5.5 Estimated bound SO₂ levels during MLF with 12 commercial strains of *O. oeni*. Bound SO₂ were calculated based on the concentration of SO₂ binding compounds, the reported dissociation constants of their sulfonates, and assuming 30 mg l⁻¹ free SO₂.

Strain	Bound SO ₂ (mg l ⁻¹)				
	Before <i>O. oeni</i> inoculation	At time of malic acid depletion	1 week after malic acid depletion	2 weeks after malic acid depletion	3 weeks after malic acid depletion
R1032	130.0±10.0	152.0±12.0	33.3±5.7	28.9±1.1	25.1±0.9
R1054	149.2±19.2	136.2±11.2	38.7±1.3	31.5±1.5	28.9±1.1
R1075	142.5±2.5	106.3±2.3	33.2±3.8	27.1±2.1	23.8±2.8
R1076	136.5±4.5	128.8±8.8	29.7±0.3	26.6±1.6	23.8±0.8
R1077	138.2±5.2	34.2±15.8	25.2±1.8	22.3±0.7	20.3±0.7
R1098	140.3±20.3	120.0±5.0	44.1±8.1	22.3±0.3	18.5±0.5
R1101	138.6±5.6	131.2±13.8	65.0±7.0	25.0±2.0	19.6±1.4
R1105	123.6±16.4	107.0±8.0	28.7±1.3	25.5±0.5	23.3±0.3
R1106	137.2±4.2	90.9±10.9	28.3±0.7	27.5±0.5	24.3±2.3
R1108	140.3±10.3	118.7±8.7	25.8±7.2	22.5±2.5	20.2±1.8
R1118	132.8±2.2	89.0±12.0	29.9±11.1	26.0±4.0	22.3±1.7
R1124	129.1±7.9	70.4±14.6	28.9±5.1	25.9±2.1	22.6±0.4
Mean	136.5±2.7	107.1±6.8	34.2±2.5	25.9±0.7	22.7±0.6
Range	123.6 – 149.2	34.2 – 152.0	25.2– 65.0	22.3 – 31.5	18.5 – 28.9

5.5. Discussion

Management of SO₂ binding compounds is integral for optimal sulfite usage in winemaking. Lower SO₂ concentrations in wines can reduce adverse consumer perceptions and reactions to wine, and ensure adherence to domestic and international sulfite regulations. This study is the first to closely investigate the kinetics of SO₂ binding compounds throughout and after MLF. Twelve commercial strains of *O. oeni* showed equivalent abilities to degrade three important SO₂ binders, but the extent of their metabolism was strain dependant.

Galacturonic acid is a wine carbonyl of grape origin (Ribéreau-Gayon et al., 1998a) found at higher concentrations in wines with significant skin contact, i.e. red wines (Sponholz and Dittrich, 1985), and wines produced from botrytized grapes (Francioli et al., 1999). Galacturonic acid was not degraded by any of the *O. oeni* strains tested in this work.

α -Ketoglutaric acid is an important intermediate for amino acid metabolism in the cell (Berg et al., 2007) and it has been suggested that lactic acid bacteria (LAB) use α -ketoglutaric acid as an electron acceptor during MLF (Zhang and Ganzle, 2010). A study by Hegazi and Abo-Elnaga (1980) found that 112 LAB belonging to the genera *Leuconostoc*, *Streptococcus* and *Lactobacillus* could not degrade α -ketoglutaric acid, whereas work by Radler (1986) using *O. oeni* in wine suggested that concentrations could be decreased. In this work, all organisms tested degraded α -ketoglutaric acid. However, on average less than half of the initial α -ketoglutaric acid was degraded and strain variation was large.

In contrast to α -ketoglutaric acid, pyruvic acid levels decreased rapidly and almost completely during MLF, across all strains studied. Among the SO₂ binders tested, pyruvate was the only one whose concentration decreased prior to malic acid metabolism. The early degradation may be attributable to its significance in a number of biochemical pathways related to energy conservation by ATP production (Henick-Kling, 1993) and NAD(P)⁺ regeneration (Maicas et al., 2002; Zaunmuller et al., 2006). The transient increase of pyruvic acid levels during the latter stages of malic acid degradation may result from its formation as an intermediate during the metabolism of citrate (Hugenholtz, 1993), which has been observed to commence towards the end of MLF (Bartowsky and Henschke, 2000; Nielsen and Richelieu, 1999). Among all *O. oeni* strains studied, R1105 produced the highest transient pyruvic acid concentrations during MLF, and subsequently displayed the highest final residues.

In wine, acetaldehyde can be rapidly metabolized by *O. oeni* (Diaz Maroto et al., 2001; Mayer et al., 1976) leading to the production of ethanol and acetic acid (Osborne et al., 2000). Osborne et al. (2006) observed that acetaldehyde metabolism was simultaneous with malic acid degradation for two *O. oeni* strains tested in white wine. However, this interpretation may have resulted from infrequent samplings. In this work, the degradation of acetaldehyde followed malic acid metabolism after a definitive delay, with a rapid phase of acetaldehyde metabolism completed 4.9 days after malic acid depletion. It is possible that the initial concentrations of malic acid and acetaldehyde may also influence the relative durations of malic acid and acetaldehyde depletion. More work would be needed to elucidate this possible relationship.

Across most tested strains, the largest decrease in calculated bound SO₂ concentrations (53%) occurred within one week following malic acid depletion coinciding with significant metabolism of acetaldehyde, whose sulfonate has a very low dissociation constant ($K_d = 1.5 \times 10^{-6}$, (Ribéreau-Gayon et al., 1998a). Together with the decrease that occurred during MLF (22%), this one week delay resulted in a total reduction of 75% of the calculated bound SO₂. Waiting for an additional two or three weeks only led to a further reduction of 6% or 8% respectively.

Post-ML activity of wine LAB may cause heterofermentation of sugars and decarboxylation of amino acids, leading to wine quality degradation by the production of acetic acid (Lonvaud-Funel, 1999), biogenic amines (Gonzalez-Marco and Ancin-Azpilicueta, 2006; Martin-Alvarez et al., 2006) and citrulline, the precursor to the carcinogenic ethyl carbamate (Mira de Orduña et al., 2000). This work suggests that a one week period following malic acid depletion may be effective to reduce SO₂ binders, while still minimizing the risk of metabolic transformations that are detrimental to overall wine quality. In wines with a higher risk for such quality degradation (high pH, residual sugars), stabilization decisions should be based on the measurement of acetaldehyde or bound SO₂ levels (following bench tests with previously sulfited wine). Finding the adequate time point for stabilization with SO₂ and/or removal of residual lees is critical for both the reduction of SO₂ binders and maintenance of wine quality.

5.6. Conclusions

Recently, a new method was presented and patented, that would allow the reduction of SO₂ binding carbonyls in wines using an insoluble resin (Blasi et al., 2008; Deleuze et

al., 2007a; Deleuze et al., 2007b). The method seems to have been especially intended for wines from Sauternes, which tend to have high SO₂ binder concentrations and SO₂ requirements. So far, the method has not been scientifically scrutinized and is not permitted. The current work shows that within the currently legal winemaking tools, MLF remains likely the most effective method to achieve reduced wine SO₂ additions. Where suitable by wine type and style, MLF can be considered within the strategies to improve SO₂ utilization.

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CHAPTER 6: IMPACT OF ACETALDEHYDE BOUND SO₂ ON THE GROWTH AND METABOLISM OF *OENOCOCCUS OENI* DURING MALOLACTIC FERMENTATION

6.1. Abstract

Sulfur dioxide (SO₂) is a potent wine preservative known for its anti-oxidant, anti-enzymatic and anti-microbial activities. Its nucleophilic properties also favor its combination with carbonyl compounds in wine, such as acetaldehyde. When SO₂ is bound, most of its preservative effects are diminished. *Saccharomyces cerevisiae* can tolerate high levels of bound SO₂, whereas *Oenococcus oeni* are known to be more sensitive. This study observed the effects of purified acetaldehyde bound SO₂ additions on the growth and metabolism of eleven strains of *O. oeni* in complex media. Measurement of SO₂ and acetaldehyde were conducted using sensitive colorimetric and enzymatic methods, respectively. Acetaldehyde bound SO₂ increased mean bacterial lag times from 3.6 days in control fermentations, to 20.4 days when inoculated at bound SO₂ levels of 90 mg l⁻¹. During malolactic fermentation, metabolism of acetaldehyde bound SO₂ by *O. oeni* resulted in concomitant increases in free SO₂ concentrations. Despite low levels of free SO₂ throughout MLF, *O. oeni* exponential growth was only observed upon near depletion of acetaldehyde bound SO₂. The inhibitory effects of acetaldehyde bound SO₂ on *O. oeni* could be lessened by adapting bacteria to bound SO₂ prior to inoculation. Interestingly, the bacteriostatic effect of acetaldehyde bound SO₂ did not inhibit *O. oeni* metabolism of malic acid; highlighting the importance of increased bacterial titers to overcome difficult environmental conditions in wine. Further work is still needed to elucidate the mechanism of acetaldehyde bound SO₂ inhibition of *O. oeni*.

6.2. Introduction

The term malolactic fermentation (MLF) refers to a secondary fermentation in the production of most red and some whites wines leading to the decarboxylation of L-malic to L-lactic acid resulting in deacidification of wines (Fugelsang and Edwards, 2007; Henick-Kling, 1993). MLF may occur spontaneously, or can be induced by inoculation with commercial starter bacteria, most of which are selected among strains of *Oenococcus oeni*, a lactic acid bacterium associated with the wine environment.

Despite the availability of starters selected for their resilience towards wine stress conditions, including high ethanol and low pH levels, sluggish and stuck MLF are still encountered (Gockowak and Henschke, 2003). Other inhibitory factors for *O. oeni* such as depletion of wine nutrients by yeast (Comitini and Ciani, 2007), presence of yeast and grape derived anti-microbial compounds (Costello et al., 2003), bacteriophages (Henick-Kling et al., 1986) and sulfur dioxide (SO₂) (Larsen et al., 2003) may also decrease the success of MLF.

SO₂ is an important *O. oeni* stressor due to its potent anti-bacterial (Carreté et al., 2002) activity. The highly reactive and nucleophilic nature of SO₂ results in the formation of sulfonates, when combined with carbonyl or keto compounds in wine. Compared with free SO₂, these bound forms of SO₂ are thought to have a minor yet evident preservative activity (Larsen et al., 2003; Rankine, 1968; Ribéreau-Gayon et al., 1998).

While *Saccharomyces cerevisiae* tolerates moderate levels of bound SO₂ (Fleet, 1993; Ribéreau-Gayon et al., 1998), its effects on lactic acid bacteria (LAB) have been

reported to be more significant (Fornachon, 1963; Hood, 1983; King and Beelman, 1986; Lafon-Lafourcade and Peynaud, 1974; Larsen et al., 2003; Mayer et al., 1976) Possible effects of acetaldehyde bound SO₂ at enologically relevant concentrations on the growth, malic acid and acetaldehyde degradation of currently available *O. oeni* strains has not been given sufficient attention. For this study, acetaldehyde hydroxysulfonate was produced in a highly purified form with little free SO₂ contamination. This sulfonate was then added directly to MRS media and allowed for generation of high-resolution time course profiles of high-resolution kinetic data following growth and metabolic degradations throughout MLF.

6.3. Materials and Methods

6.3.1. Chemicals and microorganisms

All chemicals, unless cited otherwise, were of analytical grade (Thermo Fisher Scientific, Fairlawn, NJ). Eleven *Oenococcus oeni* strains were provided by Lallemend Inc. (Montréal, Canada) and were maintained by preservation in 25% (w/v) glycerol solution at -85°C.

6.3.2. Media preparation and inoculation procedure

O. oeni strains were grown in apple MRS (AMRS) medium, which is a modification of MRS medium (de Man et al., 1960) for the growth of wine LAB. It contained 55 g of Lactobacilli MRS broth (BD, Franklin Lakes, NJ) diluted in 200 ml of a commercial pure apple juice (Wegmans, NY) and 600 ml of water. The pH was then adjusted to 4.5 with 5 N NaOH solution and the final volume was adjusted to 1 liter.

At the stationary phase of growth, *O. oeni* cells were centrifuged at 2000 g for 5 minutes and reconstituted in a sodium hydrogen tartrate buffer containing, 7.5 g l⁻¹

tartaric acid, 500 mg l⁻¹ MgSO₄, 350 mg l⁻¹ MnSO₄·5H₂O and 0.005% (w/v) Tween 80, adjusted to pH 4.5 with 5 N NaOH. *O. oeni* cells were inoculated into 10 ml test tubes containing sterile MRS media at a rate of 40 mg l⁻¹ dry weight (1.0x10⁷ CFU ml⁻¹).

6.3.3. Preparation of acetaldehyde bound SO₂ and verification of free and bound SO₂ concentrations

Preparation of acetaldehyde bound SO₂ (acetaldehyde hydroxysulfonate) was based on the technique described by Shriner and Land (1941) with modifications. Specifically, a 200 ml aqueous solution of potassium metabisulfite (1M) and acetaldehyde (2M) was mixed in an ice bath for 3.0 hours. 100 ml of 95% ethanol was then added and this solution was stored at -18°C for 24 hours. The crystals formed were then filtered under vacuum using Whatman #8 filter paper and washed with 200 ml of 70% ethanol (previously chilled to -18°C). Crystals remaining on the filter pad were stored in a desiccator over sulfuric acid for at least 3 days. Crystals were then weighed and dissolved into ASTM Class I water, prepared using a water purification system (Arium 611UV, Sartorius, Germany). Verification of free and bound SO₂ concentrations were conducted colorimetrically using a commercial kit (Megazyme, Ireland). Concentrated acetaldehyde hydroxysulfonate was then sterile filtered (0.22 µm, nylon, Millipore, Ireland) and added directly to sterile MRS media.

6.3.4. Cultivation and sampling procedures

All incubations were conducted in sealed vials and held at 25°C±2°C until the completion of MLF. Aerobic sampling was carried out under sterile conditions in a biosafety hood (Sterigard Hood Type A/B3, Sanford, ME) and lead to increased dissolved oxygen in the sterile media. Anaerobic sampling was in an anaerobic

chamber (Model 2000, Coy Laboratory Products, Grass Lake, MI) and did not increase dissolved oxygen concentrations over 0.4 mg l^{-1} . After sampling all samples were immediately frozen (-18°C) for future analysis.

6.3.5. Analytical methods and statistical analysis

O. oeni growth was monitored by measurement of optical density at 600 nm using a spectrophotometer (LKB Biochrom, UK) and dry weight measures were calculated as described by Li and Mira de Orduña (2010). Malic acid and acetaldehyde were measured enzymatically using commercial test kits (Megazyme, Ireland). Student's t-tests were conducted with JMP 7.0 (SAS, North Carolina) to determine statistical significance between sample populations at the 0.05 confidence level.

6.4. Results

Figure 6.1 shows the course of dissolved oxygen, free and acetaldehyde bound SO_2 in sterile MRS media. Under aerobic conditions, the concentration of free and acetaldehyde bound SO_2 decreased steadily over time, whereas anaerobic samples were more stable. The effects of pH were not statistically significant over the concentration range tested.

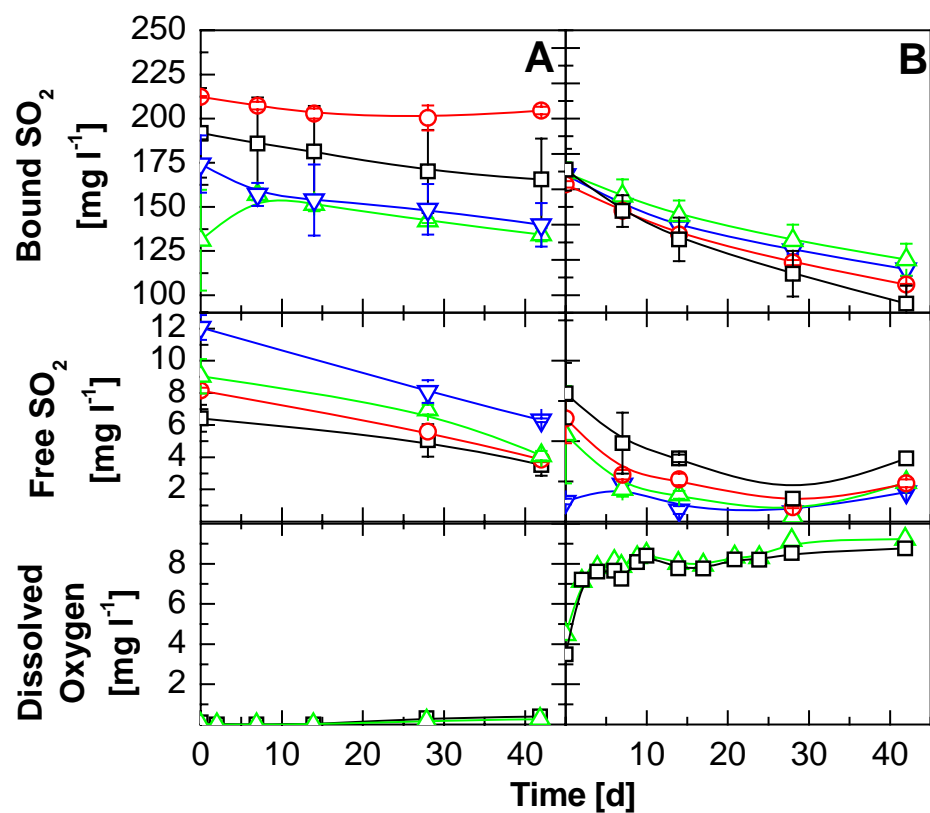


Figure 6.1 Kinetics of acetaldehyde-bound SO_2 , free SO_2 and dissolved oxygen at pH (□) 3.0, (○) 3.5, (△) 4.0 and (▽) 4.5 in MRS media under aerobic (A) and anaerobic (B) conditions.

To understand the impact of acetaldehyde bound SO₂ on the growth of *O. oeni*, eleven strains were grown in sealed vials with minimal oxygen ingress during MLF (Figure 6.2). At inoculation, free SO₂ concentrations were below 3 mg l⁻¹. Yet, a marked effect of acetaldehyde bound SO₂ on growth of *O. oeni* could be observed in all incubations. Clear differences in growth kinetics were also observed between *O. oeni* and strains were classified as “slow” or “fast” growing, accordingly (Figure 6.2).

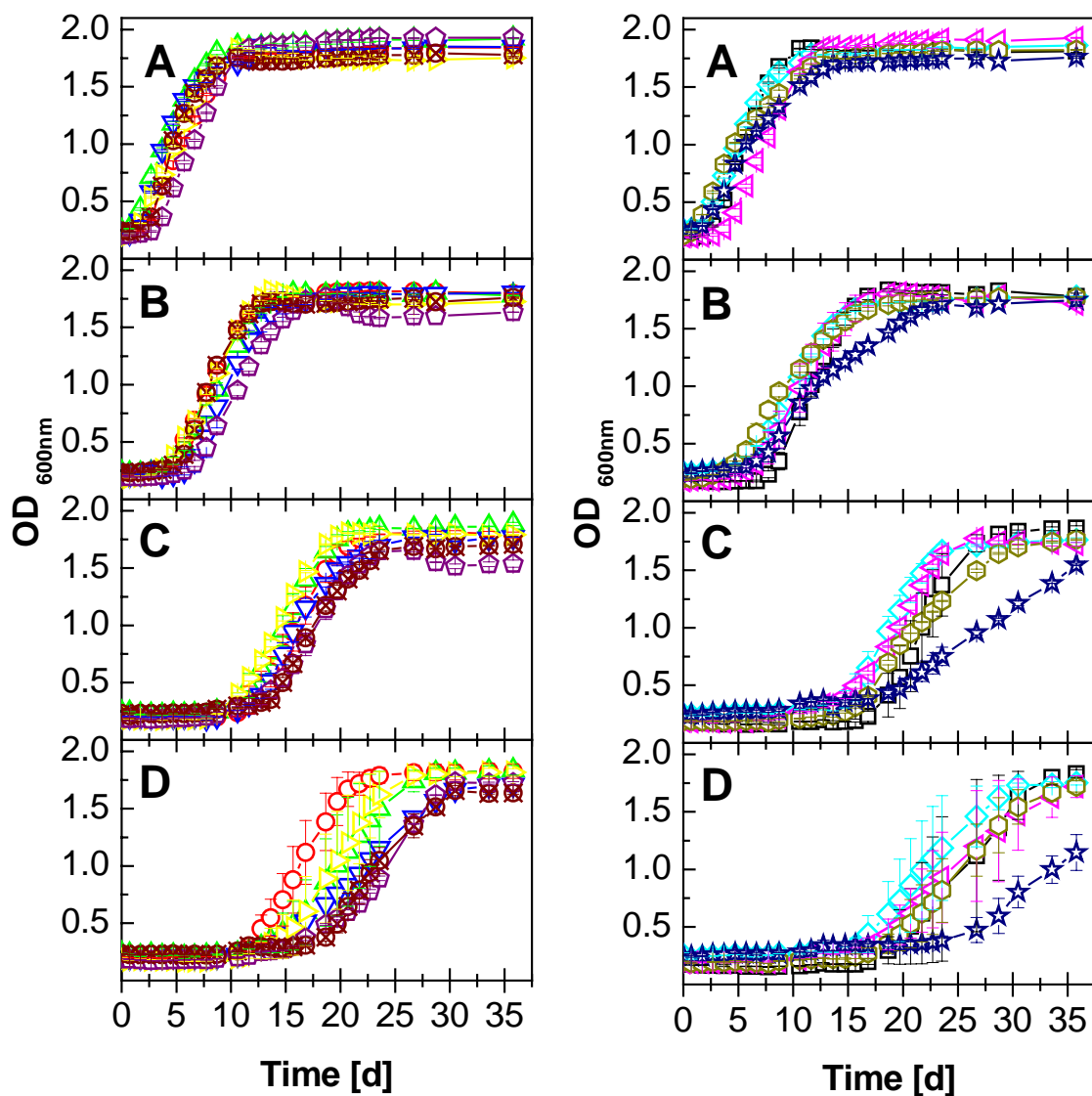


Figure 6.2 Effect of bound SO_2 at (A) 0, (B) 30, (C) 60 and (D) 90 mg l^{-1} on the growth of *O. oeni* in MRS medium at pH 4.5 under aerobic conditions. *O. oeni* strains are displayed according to their growth kinetics. “Faster” strains are shown in the left graph and include, (○) R1075, (△) R1077, (▽) R1098, (◇) R1106, (◇) R1124, (⊗) R1126, while “slower” growing strains are displayed on the right and include, (□) R1054, (◇) R1101, (◇) R1105, (◇) R1108, (☆) R1118.

Quantitatively, the strongest effect was observed for lag phases, whose increase was directly proportional to the initial bound SO₂ concentration. At 90 mg l⁻¹ the lag phase was more than five-fold compared with the control treatment (Table 6.1).

Presence of bound SO₂ also caused statistically significant decreases in maximum specific growth rates (~2 fold decrease at 90 mg l⁻¹ compared with control) and, to a lesser extent, maximum growth yields (Y_{max} values) in a dose dependant manner (Table 6.1).

To study the underlying causes for these observations, a high resolution kinetics study was conducted to investigate possible microbial degradation of acetaldehyde bound SO₂ and determine if *O. oeni* can adapt to high acetaldehyde bound SO₂ environments.

Table 6.1 Effect of bound SO₂ levels on various microbiological growth parameters recorded for eleven strains of *O. oeni* in MRS media at pH 4.5. Data was averaged across all strains. Different letters represent significant differences between treatments at $\alpha < 0.05$. Lag phase was calculated as the time needed to reach 0.6 OD_{600nm}.

Bound SO ₂ (mg l ⁻¹)	Lag phase (d)	μ Max (h ⁻¹)	Ymax (OD _{600nm})
0	3.55±0.28 ^a	0.0172±0.0016 ^a	1.854±0.017 ^a
30	7.66±0.39 ^b	0.0124±0.0009 ^b	1.793±0.008 ^{ab}
60	16.00±0.91 ^c	0.0102±0.0004 ^b	1.765±0.028 ^{bc}
90	20.38±1.15 ^d	0.0077±0.0003 ^c	1.650±0.072 ^c

Investigation of two exemplary strains of *O. oeni* confirmed that acetaldehyde bound SO₂ had a major effect on lag times and also affected the growth rates (Figure 6.3). Throughout MLF, free SO₂ concentrations remained under 8 mg l⁻¹. However, the sensitive quantification method revealed oscillations in free SO₂ concentrations during MLF. While incubations at 30 mg l⁻¹ only showed one free SO₂ peak within the first 5 days of incubations, those at higher acetaldehyde bound SO₂ concentrations showed up to two peaks, which happened later during the experiments. The free SO₂ peaks were associated with decreases in acetaldehyde bound SO₂ levels. The latter, in turn, were associated with degradation of acetaldehyde. In both *O. oeni* inoculated fermentations studied, acetaldehyde bound SO₂ concentrations decreased steadily over time at rates similar to uninoculated aerobic control samples. However, increased rates of acetaldehyde bound SO₂ degradation were observed near the end of MLF.

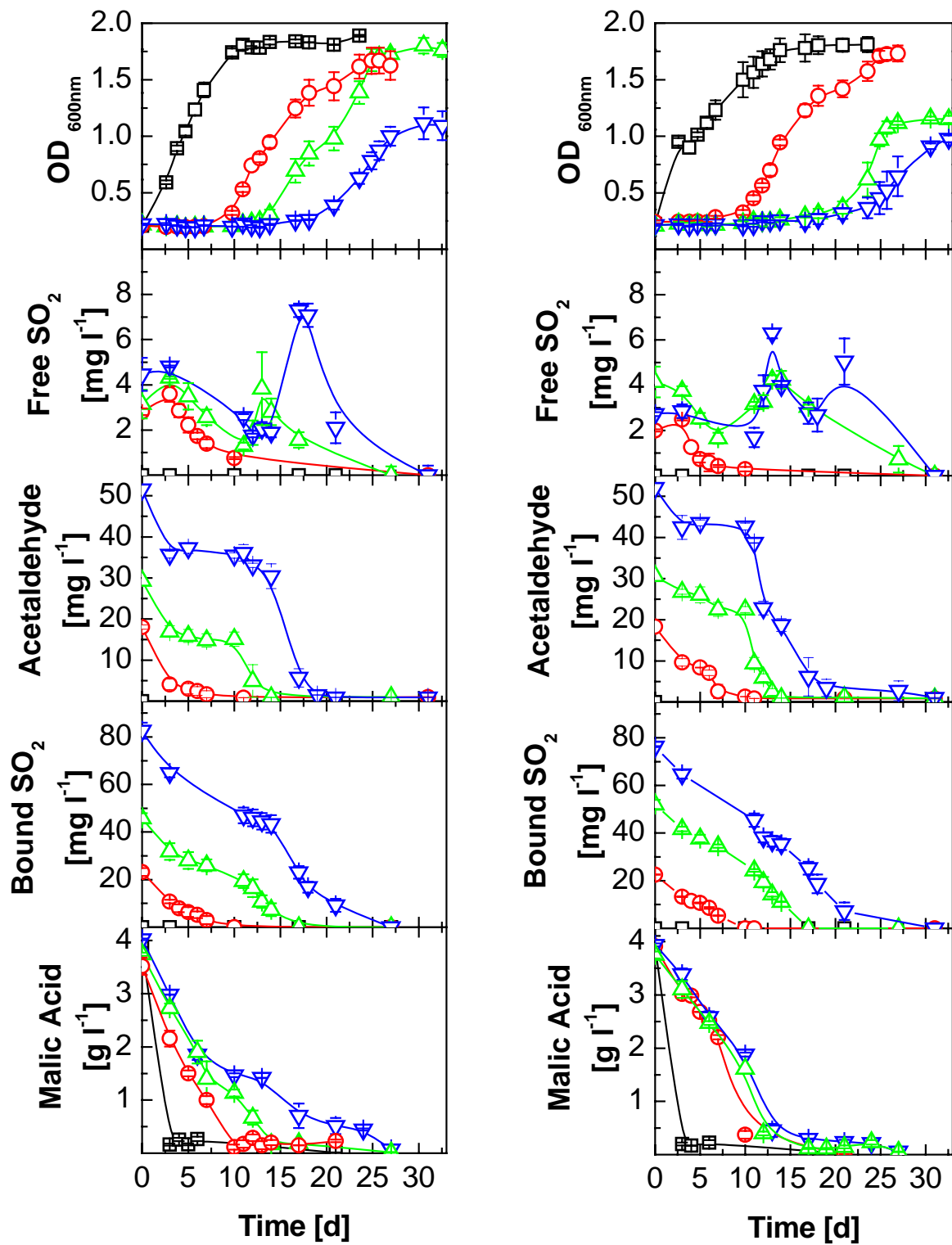


Figure 6.3 Effect of bound SO_2 at (\square) 0, (\circ) 30, (\triangle) 60 and (∇) 90 mg l^{-1} on the growth and metabolism of *O. oeni* strains (L) R1077 and (R) R1118 in MRS media at pH 4.5.

Despite low residual levels of free SO₂ in the MRS media during MLF, significant bacterial growth commenced slightly before the depletion of acetaldehyde bound SO₂. *O. oeni* growth commenced at higher residual free and bound SO₂ concentrations in treatments with higher initial concentrations of bound SO₂ (Table 6.2).

Table 6.2 Concentration of free, bound, total SO₂ and acetaldehyde at the beginning of the exponential growth phase. Three treatments are compared with varying levels of initial acetaldehyde bound SO₂ in MRS media at pH 4.5. Different letters represent significant differences between treatments at $\alpha < 0.05$.

Initial	Strain R1077					Strain R1118		
	Free	Bound	Acetaldehyde	Total	Free SO ₂	Bound	Acetaldehyde	Total
SO ₂	SO ₂	SO ₂	(mg l ⁻¹)	SO ₂	(mg l ⁻¹)	SO ₂	(mg l ⁻¹)	SO ₂
(mg l ⁻¹)	(mg l ⁻¹)	(mg l ⁻¹)		(mg l ⁻¹)		(mg l ⁻¹)		(mg l ⁻¹)
30	1.1±0.1 ^a	2.2±0.3 ^a	3.74±3.20 ^a	3.3±0.4 ^a	0.6±0.2 ^a	3.1±1.2 ^a	1.50±1.11 ^a	3.7±1.4 ^a
60	3.2±0.6 ^a	9.4±0.9 ^b	1.24±1.65 ^a	12.6±1.5 ^b	2.9±0.5 ^{ab}	5.8±2.7 ^a	0.93±1.37 ^a	8.7±3.2 ^b
90	4.4±0.4 ^b	13.7±1.3 ^c	2.88±0.82 ^a	18.1±1.6 ^c	3.8±0.6 ^b	13.7±2.9 ^b	1.34±0.86 ^a	17.4±2.3 ^b

Malic acid degradation began soon after inoculation regardless of bacterial growth, but the rate of its metabolism by *O. oeni* was inversely proportional to acetaldehyde bound SO₂ levels (Figure 6.3). For example, *O. oeni* strain R1077 had degraded 96.7%, 95.5% and 88.9% of the malic acid at the onset of exponential growth in treatments with initial bound SO₂ concentrations of 30, 60 and 90 mg l⁻¹, respectively.

Collecting stationary phase *O. oeni* and reinoculating them into medium with the same initial concentration of acetaldehyde bound SO₂ showed adaptive capabilities for *O. oeni*. This resulted in a decrease in lag time upon subcultivation into the same medium (Figure 6.4). For *O. oeni* strain R1077, statistically significant differences in lag phase times from sequential incubations in the same medium were obtained, whereas strain R1124 did not show the same degree of adaptation (Table 6.3).

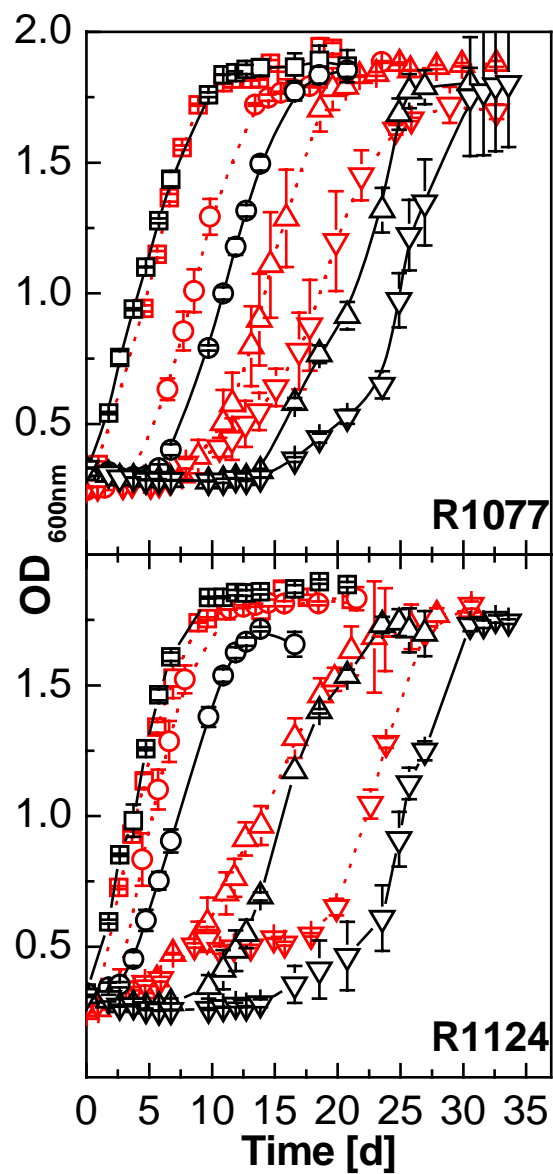


Figure 6.4 Effect of subcultivation on the growth of *O. oeni* strain R1077 and R1124 when incubated with different bound SO_2 levels, (\square) 0 mg l^{-1} , (\circ) 30 mg l^{-1} , (\triangle) 60 mg l^{-1} and (∇) 90 mg l^{-1} . (—) 1st growth generation, (.....) subcultivation from 1st generation after cells have reached stationary phase.

Table 6.3 Effect of bound SO₂ on the lag phase of *O. oeni* growth in MRS media over two generations.

Bound SO ₂ (mg l ⁻¹)	R1077			R1124		
	Lag Phase (d)			Lag Phase (d)		
	1 st Growth gen.	2 nd	P-value	1 st Growth gen.	2 nd Growth	P-value
		Growth gen.			gen.	
0	1.8±0.0	1.0±0.0	N/A	1.8±0.0	1.0±0.0	N/A
30	5.8±0.0	2.1±0.6	0.0249	3.7±0.0	2.1±0.6	0.1108
60	13.9±0.0	5.8±1.1	0.0194	7.7±2.0	3.1±0.5	0.1524
90	13.9±0.0	7.3±1.3	0.0390	13.2±3.4	9.6±0.0	0.4084

6.5. Discussion

SO₂ is a potent anti-microbial and its inhibition of *Saccharomyces cerevisiae* and *Oenococcus oeni* has been well described (Hinze and Holzer, 1986; Rehm et al., 1964; Ribéreau-Gayon et al., 1998; Schimz and Holzer, 1979). However, bound SO₂ concentrations in wine are often overlooked due to perceptions of its impaired activity compared to free SO₂. Despite this fact, wine LAB are still sensitive to bound SO₂ (Fornachon, 1963; Larsen et al., 2003) and this may impact the course of MLF in wines. Currently, little is known about the fate of bound SO₂ and its metabolites during MLF with *O. oeni*. This study is the first to provide high-resolution kinetic profiles of acetaldehyde bound SO₂ and its effects on the growth and metabolism for eleven strains of *O. oeni* in a complex, reproducible medium.

Declining concentrations of acetaldehyde bound SO₂ in aerobic environments prompted the implementation of containers with airtight seals and brief sampling times during MLF. This led to a slow but steady decrease of free and acetaldehyde bound SO₂ levels in the media, and was also confirmed in wine by work from Fell et al. (2007). Increased oxygen concentrations in media can increase chemical oxidation reactions (Danilewicz, 2007), leading to a decrease in free SO₂ and a loss of bound SO₂ due to an equilibrium shift.

Decreases in acetaldehyde bound SO₂ from oxygen ingress should have been minimal during growth studies of eleven *O. oeni* strains in MRS media, due to tight seals on fermentation containers. Under these conditions, increased lag phase, combined with decreased growth rates and yields were noted for *O. oeni* and present challenges for successful/efficient MLF. In many wineries it is common practice to inoculate a

commercial strain of LAB with known characteristics and strong fermentation kinetics (Fugelsang and Edwards, 2007). Inhibition of a selected bacteria strain will result in sub-optimal tank utilization in wineries, and increases the probability of wine spoilage (Davis et al., 1988), since wine cannot be fully stabilized prior to the completion of MLF.

It has been hypothesized that bacterial inhibition by bound SO₂ involves the liberation of free SO₂ from the sulfonate upon degradation of the carbonyl or keto moiety by wine LAB. (Hood, 1983; Lafon-Lafourcade and Peynaud, 1974; Somers and Wescombe, 1987). This is plausible since wine LAB are known to degrade various aldehydes that bind to SO₂ (Diaz Maroto et al., 2001; Osborne et al., 2000). This study is the first to conclusively show that free SO₂ is released by *O. oeni* during metabolism of acetaldehyde bound SO₂, using sensitive colorimetric and enzymatic methods for the measurement of SO₂ and acetaldehyde, respectively. However, low concentrations of free SO₂ encountered throughout MLF, combined with the relatively high pH (4.5) of the media, raises questions about the anti-bacterial nature of free SO₂ under these conditions. Accordingly, other authors, have suggested that bound SO₂, itself, causes bacterial inhibition (Fornachon, 1963; King and Beelman, 1986; Larsen et al., 2003; Mayer et al., 1976). The findings highlight the need for further investigation into possible anti-microbial activities of acetaldehyde bound SO₂.

Despite growth inhibition by acetaldehyde bound SO₂, *O. oeni* cells began to degrade malic acid shortly after inoculation. Work by Lonvaud-Funel (1999) suggested that MLF would commence when *O. oeni* populations reached 10⁶ CFU ml⁻¹. In this study, cells were inoculated at 10⁷ CFU ml⁻¹ and this likely explains the rapid metabolism of

malic acid. Practical applications from this finding suggest that high bacterial inoculations into wines with high bound SO₂ levels may be effective for the deacidification of wines even under difficult conditions.

While acetaldehyde bound SO₂ significantly inhibited the growth of all bacterial strains tested, *O. oeni* did show adaptive abilities toward bound SO₂. A significant decrease in strain R1077's lag time after growth in media containing bound SO₂, highlights the ability of some *O. oeni* to adjust to these harsh environmental conditions. *O. oeni* adaptation may also explain why cells inoculated with higher concentrations of acetaldehyde bound SO₂ were able to begin their exponential growth phase with higher levels of both free and bound SO₂ in the medium, as compared to those inoculated with lower levels of bound SO₂. Further study is needed to determine underlying reason for “fast” and “slow” growth kinetics in studied *O. oeni* strains.

6.6. Conclusions

High-resolution time course profiles of malic acid, acetaldehyde and free and bound SO₂ concentrations demonstrate inhibition of *O. oeni* growth by purified additions of acetaldehyde bound SO₂. *O. oeni* could metabolize the acetaldehyde moiety of bound SO₂, concurrently liberating free SO₂. Nevertheless, the duration of *O. oeni* lag phase showed strong association with acetaldehyde bound SO₂ concentrations, warranting further study into its effects on LAB cells. Interestingly, malic acid was degraded by *O. oeni* despite the presence of acetaldehyde bound SO₂, however its rate of metabolism was directly related to bound SO₂ levels. Findings highlight the use of high bacterial inocula and adapted *O. oeni* cells as possible solutions for the completion of MLF under difficult conditions.

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CHAPTER 7: GENERAL CONCLUSIONS

The work presented in this dissertation has evaluated strategies to increase the efficacy of sulfur dioxide (SO₂) during vinification through management of SO₂ binding compounds. The following discussion aims to summarize important research findings and provide suggestions for the production of wines with low SO₂ demands.

The potency of SO₂ is highest when it is in a free or unbound state. High concentrations of carbonyl or keto compounds in wine, especially acetaldehyde, can bind to SO₂ greatly diminishing its efficacy. Hence, increased SO₂ additions are needed to achieve equivalent preservative action. However, adding more sulfites to wine may exceed domestic or international regulatory limits. In addition, a small subset of the population are known to be hypersensitive to sulfites, leading to adverse health effects upon consumption of high sulfite wines. Accordingly, consumer interest in wines with low sulfite concentrations continues to grow, although producing these wines can be challenging without proper understanding of SO₂ binding compounds. Hence, this dissertation investigated strategies for the management of important SO₂ binders including, glucose, galacturonic acid, acetoin, α -ketoglutaric acid, pyruvic acid and acetaldehyde throughout the process of vinification.

The need for comprehensive analysis of SO₂ binding compounds in wines lead to the development of a novel UHPLC method that used the metal chelator EDTA to control *de novo* formation of acetaldehyde during sample preparation. This method greatly improved existing protocols that were prone to sample oxidation and limited by

lengthy and complex sampling procedures. Implementation of this method in a commercial wine setting will help identify optimal sulfite dosing requirements based on SO₂ binder pools in wine.

The newly developed method was then used to determine the SO₂ binder concentration in over 230 commercial wines from across NYS. Results showed clear differences in the SO₂ binder profiles between red and white wines. Red wines were typically higher in α -ketoglutaric acid and galacturonic acid, whereas, white wines were higher in acetaldehyde, pyruvic acid and glucose. Differences in the SO₂ binder content between the two wine types likely reflect fundamental differences in processing (e.g. skin contact, pectinase usage) and wine style (e.g. residual sugar, malolactic fermentation). Overall, mean calculated levels of bound SO₂ in red and white wines in the study were 66 mg l⁻¹ and 81 mg l⁻¹, respectively. The higher level of bound SO₂ in white wines reflects higher concentrations of acetaldehyde therein. Based on wine carbonyl concentrations and dissociation constants with SO₂, acetaldehyde was found to be the most important SO₂ binder, accounting for 72% of bound SO₂ in white wines and 56% in reds.

The degree of acetaldehyde binding of SO₂ in NYS wines prompted further study of its formation during alcoholic fermentation (AF) by *Saccharomyces cerevisiae*. SO₂ addition prior to AF significantly increased acetaldehyde levels by binding acetaldehyde needed by *S. cerevisiae* as an electron acceptor. Further increases in acetaldehyde were due to sluggish AF conditions, possibly from micronutrient

deficiencies in grape must and from cooler AF temperatures, which limited *S. cerevisiae* reuptake of acetaldehyde. Avoidance of SO₂ additions prior to AF and selection of good quality grapes are good winemaking practices that can help keep acetaldehyde levels to a minimum during AF.

With the exception of galacturonic acid, the concentrations of acetaldehyde, pyruvic acid and α -ketoglutaric acid decreased following inoculation with *O. oeni*. On average, the end of the rapid pyruvic acid degradation phase was found to occur 3 days before the depletion of malic acid, whereas for acetaldehyde concentrations it was 4.9 days after. Overall bound SO₂ levels were decreased by 22% during MLF. However, additional wine contact with the bacterial lees resulted in a further bound SO₂ decrease of 53%. Hence, stabilization of wines immediately after malic acid depletion may limit SO₂ binder metabolism by *O. oeni*, resulting in higher wine SO₂ requirements.

Failure to control SO₂ binding compounds and bound SO₂ in particular may hinder the production of quality wine. Specifically, high levels of bound SO₂ are known to be inhibitory to *O. oeni*, possibly resulting in a sluggish or stuck MLF. Acetaldehyde bound SO₂ increased mean bacterial lag times in a dose dependant manner. During MLF, metabolism of acetaldehyde bound SO₂ by *O. oeni* resulted in concomitant increases in free SO₂ concentrations. Yet, the timing of bacteria exponential growth was closely associated with a decrease in bound SO₂ levels and suggests that bound SO₂ itself is responsible for bacterial inhibition and not free SO₂ as previously

hypothesized. Findings support the use of large inocula and adapted *O. oeni* cells as possible solutions for the completion of MLF under difficult conditions.

After AF and MLF, the effects of cellaring also play an important role in the regulation of acetaldehyde. A 2-year study involving 8 NYS wineries highlighted critical control points for acetaldehyde formation throughout the vinification process. In the 2009 vintage, acetaldehyde was found to increase during aging and bottling operations, possibly due to oxygen ingress leading to chemical oxidation of wines. This information was reported back to the participating wineries with remedial suggestions and also presented at regional winemaking conferences. Results from the 2010 vintage showed that most winemakers had improved their control of acetaldehyde during vinification, with an average decrease in acetaldehyde concentrations of 50% compared to the previous year. This finding demonstrated that through educational intervention, concepts devised and tested on the laboratory scale could be successfully transferred to commercial winemaking settings.

In summation, the management of SO₂ binding compounds, and acetaldehyde in particular, is an ongoing task throughout the entire vinification process. As grapes come into the winery their quality should be inspected to ensure lack of rot, which can increase SO₂ binder concentrations. Fungicidal residues should also be avoided as this may lead to a stuck/sluggish fermentation and increase acetaldehyde levels. Pectinase and SO₂ use on grapes should be avoided if possible, as these practices can increase galacturonic acid and acetaldehyde concentrations, respectively. Increased temperature during the latter stages of AF combined with extended yeast lees contact and oxygen

exclusion should encourage maximal re-uptake of acetaldehyde by *S. cerevisiae*. If MLF is desired after AF, higher inoculation rates and should be considered if bound SO₂ levels are high. Optimal reduction of SO₂ binders during MLF requires careful monitoring of acetaldehyde, which is typically degraded after malic acid depletion. Post-fermentation, the exclusion of oxygen from wine, especially white wine, is critical for the prevention of *de novo* formation of acetaldehyde from ethanol oxidation. Aging and bottling operations should be intermittently monitored with an oxygen-measuring device if possible.